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Exosomes from lung and breast milk – regulators of immune responses

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EXOSOMES FROM LUNG AND BREAST MILK - REGULATORS OF IMMUNE RESPONSES

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ABSTRACT

Exosomes are key mediators of intercellular communication with the capacity to regulate immune responses, locally and distally. Most knowledge on exosomal function has been gained from exosomes generated from *in vitro* cell cultures, which has helped define the role of exosomes from specific cell types. However, studying *ex-vivo* isolated exosomes from body fluids are more likely to yield clues about their *in vivo* role. This thesis aimed to gain knowledge regarding the role of human-derived exosomes from two important organs with a well-equipped immune system, namely the lung and the mammary gland.

In **study I** we studied exosomes from bronchoalveolar lavage fluid (BALF) of patients with pulmonary sarcoidosis and healthy individuals. We detected elevated levels of exosomes with an altered exosome protein profile in BALF from patients compared to exosomes from healthy controls. Exosomes isolated from patients exhibited pro-inflammatory functions seen by their ability to induce inflammatory cytokine release (IFNγ and IL-13) in autologous peripheral blood mononuclear cells (PBMCs) and (IL-8) in the bronchial epithelial cell line (BECs) 16HB14. These data suggest that exosomes may contribute to the inflammatory state of sarcoidosis.
In order to investigate the specificity of the findings in study I relative to other inflammatory settings, we performed analysis of BALF exosomes from asthmatics. In **study II** we found that several surface proteins (CD36, CD63 and CD81) were upregulated on BALF exosomes from asthmatics during steady state and after allergen challenge compared to healthy controls. In addition, BALF-derived exosomes from asthmatics at steady state and after allergen challenge could stimulate secretion of leukotrienes (LTs) and IL-8 in BECs, suggesting a pro-inflammatory function of BALF exosomes in asthmatic inflammation.
Breastfeeding is associated with health benefits for the child with possible effects on allergy development. Variations in immune-composition in breast milk between mothers could potentially result in differences in allergic outcome. Therefore, in **study III** we sought to determine whether maternal sensitization and lifestyle could affect exosome composition in breast milk. Accordingly, we found that both maternal immune status and environmental factors have a differential impact on subpopulations of exosomes in breast milk with potential effects on allergic outcome of the child as a consequence.
In **study IV** we aimed to study the effect of breast milk-exosomes on HIV infection based on findings suggesting a protective role of breastfeeding on HIV infection from mother-to-child. We report a protective role of human breast milk exosomes on HIV infection of dendritic cells and subsequent transfer and infectivity of T cells. Thus, we suggest that breast milk-exosomes might constitute one protective factor in milk against HIV infection in the child.
In conclusion, this thesis provides important insights into the composition and function of exosomes from human BALF and breast milk. Furthermore, it sheds light on how different immunological conditions can perturb the function and phenotype of exosomes *in vivo*. In the future, this could have important implications for the development of novel treatments against inflammatory diseases using exosomes as targets or therapeutic vehicles.

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To my mother Maria José and my daughter Isabel 栄美子

"Great things are done by a series of small things brought together"
- Vincent Van Gogh -

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
16HB14o	16 human bronchial 14o epithelial cell line
AHR	Airway hyperesponsiveness
ALADDIN	Assessment of Lifestyle and Allergic Disease During INfancy
AM	Alveolar macrophages
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BECs	Bronchial epithelial cells
CD	Cluster of differentiation
CLRs	C-type lectin receptors
$cPLA_2$	Cytosolic phospholipase A_2
CTLs	Cytotoxic T lymphocytes
CysLTs	Cysteinyl leukotrienes
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ECs	Epithelial cells
EM	Electron microscopy
ESCRT	Endosomal sorting complex required for transport
FLT3L	Fms-related tyrosine kinase 3 ligand
Foxp3	Forkhead box protein 3
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High-performace liquid chromatography
HSP	Heat shock protein
iDCs	Immature dendritic cells
IECs	Intestinal epithelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LNs	Lymph nodes
LTs	Leukotrienes
LTA_4	Leukotriene A_4
LTA_4H	Leukotriene A_4 hydrolase
LTB_4/C_4	Leukotriene B_4/C_4

LTC_4S	Leukotriene C_4 synthase
MDDCs	Monocyte-derived dendritic cells
MHC	Major histocompatibility complex
MVBs	Multivesicular bodies
NRG1	Neuregulin-1
OVA	Ovalbumin
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PRRs	Pathogen-recognition receptors
RA	Retinoic acid
RNA	Ribonucleic acid
TCR	T cell receptor
TF	Transcription factor
TGFβ	Transforming growth factor-β
Th	T-helper
TLRs	Toll-like receptors
Tregs	T regulatory cells

1 INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

Life on earth began more than 3.5 billion years ago [1]. Very early on, unicellular organisms already possessed an innate defense system capable of recognizing and destructing pathogens (virus, bacterium, prion, fungus or protozoan that cause disease), as well as the ability to discriminate between self and non-self. This effective, although limited immune defense still dominates in most organisms, and it was not until 500 million years ago that a sophisticated and highly diverse adaptive immune system emerged in jawed vertebrates [1]. The acquisition of such evolutionary advanced system has arguably favored a significant survival advantage against lethal pathogens. Nevertheless, the trade-offs of having a specific immune system can be of significant cost: propensity for immune attack against itself (autoimmune disease). Thus, high levels of immune responses against pathogens need to be tightly regulated in order to avoid self-destruction. To achieve this, a delicate inter-cellular communication is of crucial importance to successfully co-ordinate the innate and adaptive arm in order to maintain a functional immune system. In this section I will focus on new developments in the field of mucosal immunology.

1.1.1 INNATE DEFENSES

The innate immune system is the first line of host defense against life threatening insults. It is often defined as being non-specific in their ability to recognize antigens – an erroneous concept since it possesses a certain degree of specificity based on its potential to discriminate between pathogens, commensal microbes and self-antigens. Body surfaces, in particular mucosal surfaces, are constantly exposed to microbes and are often the port of entry for intruders [2]. However, upon barrier penetration, pathogens are instantly encountered by an array of specialized innate immune cells carrying pathogen-recognition receptors (PRRs), which can sense and bind conserved molecular patterns found on microbes, but not on mammalian cells. Sensing of such motifs by PRRs initiate pro-inflammatory signaling pathways in order to help combat infection [3].

Mucous membranes - including the gastrointestinal, respiratory, reproductive and urinary tract - are in close interaction with the external environment [2] and are mostly composed of a single monolayer of epithelium. Therefore, mucosal tissues rely on additional defense mechanisms to ensure barrier protection, including mucus secretion and a particularly strong innate immune system.

1.1.1.1 Mucus barrier

Protection against infections at mucosal sites relies to a great extent on the barrier properties of the epithelial cells with its overlaying mucus layer [4]. Specialized mucus-producing epithelial cells, known as goblet cells, generate two layers of mucus; a loose and viscous layer of secreted (gel-forming) mucins (e.g. MUC2, MUC5AC and MUC5B)

and an inner layer composed by densely packed membrane-bound mucins with a "bottle-brush appearance" (e.g. MUC1, MUC4 and MUC13). The textbook view that mucus acts as an adhesive surface that arrests microbial motility, has recently been challenged by studies showing that mucus actually promotes bacterial movement [5]. By maintaining microbes in a "free swimming state" it prevents bacteria from aggregating and generating biofilm formation that would otherwise help mediate bacterial infiltration and infection.

Both gut- and respiratory mucosa prevents inflammation by establishing a barrier against bacteria and food antigens. Tissue distribution of mucins, however, differs between the gut and the lung with MUC2 being the most abundant gel-forming mucin in the gut, while MUC5AC and MUC5B predominate in the airways. Recent findings by Shan *et al* found that MUC2 in the gut can deliver tolerogenic signals to DCs in the lamina propia [6], thus preventing DCs from mounting inflammatory-responses against innocuous antigens. Furthermore, gastrointestinal mucus can inhibit colitis and growth of *Helicobacter pylori* [7, 8], reflecting the importance of mucus against gastrointestinal infections. In the airways, MUC5B has been shown to play indispensable roles by mediating removal of harmful material trapped in the mucus barrier through the rhythmic beat of cilia (mucociliary clearance). In fact, mice deficient in MUC5B have an accumulation of material in upper and lower airways leading to chronic infection [9], supporting the requirement of MUC5B to maintain airway homeostasis.

Paradoxically, although mucins function as steric hindrance against microbes, it also constitutes an attachment point for pathogens to initiate cell invasion. Therefore, shedding of membrane-bound mucins into the extracellular milieu could mediate a protective mechanism against bacterial attachment and subsequent infection. In fact, binding of *H. pylori* to the cell-anchored MUC1 on intestinal epithelial cells (IECs) have been shown to increase shedding of the mucin from the surface [10]. Additional studies have shown that gastric epithelial cells expressing MUC1 bind *H. pylori* to a less extent compared to MUC1-/- cells [11], which in part can be explained by the function of MUC1 as a "shedding receptor". The protective effect of MUC1 against infections is best illustrated in MUC1 deficient mice, which exhibit increased susceptibility to *H. pylori* infection compared to wild type (WT) counterparts [11]. However, respiratory *Pseudomonas aeruginosa* infection in MUC1 deficient mice have instead increased clearance of bacteria in the airways compared to WT mice [12]. In this context, MUC1 was able to function as an anti-inflammatory signaling molecule and inhibit TLR5-mediated activation of nuclear factor (Nf)-κB. Thus, it appears that MUC1 works in several ways, including steric- and decoy receptor functions, in addition to its anti-inflammatory signaling properties.

1.1.1.2 Epithelial barrier and microbial sensing

Although most microbes do not generally pass the mucus layer, it happens that pathogens succeed to penetrate and reach the epithelium. Pathogen invasion into the tissue often involves attachment to the epithelial surface and subsequent internalization. Based on the exposed location of mucosal epithelial cells (ECs), in

particular the airways and more so in the gut, ECs are equipped with PRRs able to sense and respond to commensals and pathogens expressing conserved motifs (pathogen-associated molecular patterns (PAMPs)) and molecules from damaged tissue (damaged-associated molecular patterns (DAMPs)) [13]. ECs express various PRRs, including Toll-like receptors (TLRs), which upon stimulation associate with a multitude of adaptor proteins (e.g. myeloid differentiation primary response protein (MyD)88, Myd88 adaptor-like (Mal), Toll/IL-1 receptor domain-containing adaptor inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM)) [13]. Signaling events propagated by these adaptor proteins generally result in the activation of Nf-κB or the interferon regulatory factors 3 and 7 (IRF-3 and IRF-7) that leads to the expression of pro-inflammatory cytokines and type I interferons [14]. To date, TLR2 and TLR4 are the TLRs that have been most extensively studied. Activation of TLR4 signaling occurs when binding to the bacterial component lipopolysaccharide (LPS) expressed by gram-negative bacteria [15], whereas TLR2 responds to bacterial lipoproteins from gram-positive bacteria [16], yeast and mycobacteria [17].

Human ECs in the lung and the intestine express large numbers of TLRs [18, 19]. However, expression levels and location of specific TLRs on ECs vary depending on the anatomical location and the degree of microbial exposure at the given site. Based on the constant exposure of ECs to PAMPs in the gut lumen, intestinal epithelial cells (IECs) have developed different strategies to avoid chronic TLR–stimulation. For instance, expression of TLR2 and TLR4 are confined to the basolateral side of human fetal enterocytes, and are aberrantly located at the apical side in patients with Crohn's disease [18]. This suggests that a polarized distribution of TLRs on IECs might be an important mechanism to avoid inflammation.

Notably, TLR-signaling on ECs constitutes an important communication bridge between the external world and the immune cells located at the basolateral side of the ECs. For instance, Hammad *et al* showed that TLR4 expression on airway epithelial cells, but not on hematopoietic cells, are necessary and sufficient to achieve recruitment, activation and migration of mucosal DCs in response to inhaled endotoxin and to achieve allergen induced asthma in response to house dust mite [20].

1.1.1.3 Communication of mucosal ECs with innate immune cells

Over the past decade, major advances have occurred in our understanding of the crucial role of mucosal epithelium to maintain immune homeostasis and regulate immune reactions [21]. ECs are the first cells encountering environmental stimuli and play an important role in the cross-communication between the external world and immune cells located underneath the epithelium. The immune cells that have received most attention in this regard are dendritic cells (DCs), monocytes, macrophages and the recently identified group of cells named innate lymphoid cells (ILCs) [22]. However, ECs can also influence other innate cells, including mast cells, eosinophils and basophils [19].

1.1.1.4 Dendritic cells

DCs, a type of professional antigen-presenting cells (APCs), are the "commanders-in chief" of the immune system. They are responsible for connecting the innate and adaptive immune system, and determine inflammatory or tolerogenic outcome and magnitude of the final immune response. During steady state, most DCs are located in the tissue in an immature state where they sample the environment for antigens via phagocytosis [23]. Upon recognition of pathogens or by sensing pro-inflammatory cytokines, DCs will mature and migrate from the periphery to secondary lymphoid organs where they present antigens to, among others, T cells. In addition, DCs provide appropriate co-stimulation in order to differentiate and boost the expansion of antigen-specific T cells.

DCs sense and react to microbes through multiple PRRs expressed endosomally and at the cell surface. One such group of receptors are the C-type lectin receptors (CLRs), including DC-SIGN, DEC205 and Dectin 1, which recognize carbohydrate domains on a variety of pathogens [24]. Recently, it has become evident that several pathogens, including human immunodeficiency virus (HIV), target DC-SIGN to modulate DC-biology by interfering with TLR signaling [25] in order to avoid destruction and promote infection [26]. In fact, binding of the HIV protein gp120 to DC-SIGN leads to productive infection of DCs [27] and efficient *trans*-infection of T cells [28].

Capturing of harmless material by DCs is believed to induce antigen-specific tolerance whereas sensing of potentially dangerous microbes leads to inflammatory responses [29]. DCs in the skin and at mucosal sites need to cope with the challenge of balancing inflammatory responses against pathogens with tolerance against beneficial commensals. This is particularly so for DCs in the gut and lungs. It has been postulated that the actual tissue microenvironment conditions DC function rather than the existence of functionally different DC subsets [30]. This is partly illustrated by the functional differences between DCs from anatomically different sites. For instance, the small intestine contains high levels of retinoic acid (RA) (vitamin A derivate), which confers the ability of intestinal DCs to produce its own RA and the capacity to imprint gut-tropic T-regulatory cells (Tregs) (cells with the ability to dampen the immune response) [31]. However, this function can be abolished *in vivo* by depriving mice from vitamin A causing RA to be eliminated from the intestine. In contrast, splenic DCs with no ability to produce RA can be conditioned to do so by exposing them to a RA-rich environment. Furthermore, DCs can be conditioned and functionally monitored by microbes and/or by cytokines in the environment [32], which is unique to the given tissue.

1.1.1.5 Dendritic cell subtypes and division of labor

Although the tissue microenvironment plays a crucial role in shaping DC function, other factors including life history of DCs (migratory, resident, circulating), host state (steady state or inflammation) and activation and maturation state of the DCs, are likely involved in dictating DC function and final immune response [33]. In addition, DC

function can also be attributed to the existence of functionally different DC subtypes. In fact, several subclasses have been identified in both mice and humans with functional specializations in the generation of immune responses [34].

DCs can be broadly divided into two main subtypes: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). During steady state, cDC progenitors egress from the bone-marrow (BM) and migrate through the blood until reaching peripheral tissues and lymphoid organs, followed by differentiation of distinct DC subtypes in situ [35]. DCs at peripheral tissues are constantly patrolling to the draining LNs where they can initiate immune responses. However, distinct subpopulations of DCs can be found at different peripheral tissues. As an example, in the murine system there are at least two separate subpopulations of DCs in the intestine, which are defined by the expression of CD103 and CD11b ($CD11c^{hi}CD103^{+}CX3CR1^{-}CD11b^{-}$, $CD11c^{hi}CD103^{+}CX3CR1^{-}CD11b^{+}$) [36]. Although a third population expresses high CD11c levels ($CD11c^{hi}CD103^{-}CX3CR1^{+}CD11b^{+}$), they are now considered macrophages due to their poor ability to migrate and prime T cells, among other characteristics. Similarly, two classes of cDCs have been found in the lung ($CD103^{+}$ and $CD11b^{hi}$) [37], each with distinct and overlapping roles in antigen presentation. In the draining LNs of lung and gut, migratory DCs can be distinguished from resident DCs by the expression of CD103. In contrast to migratory DCs, resident DCs ($CD4^{+}CD8\alpha^{-}CD11b^{+}$ and $CD4^{-}CD8\alpha^{+}CD11b^{+}$) stay in the draining LN throughout their life span in an immature state. The $CD8^{+}$ population is specialized in cross-presentation of dying cells and bacteria while $CD8^{-}$ cells are more efficient in MHC class II presentation.

In humans, less is known about DC subtypes and function. Nevertheless, three separate subtypes of DCs have been identified in blood: pDCs and two subsets of myeloid DCs (equivalent to mouse cDCs) ($BDCA1^{+}$ and $BDCA3^{+}$), the latter shown to correspond to LN-resident DCs [38]. In addition, several migratory subtypes of DCs with distinct abilities to mount T cell responses have been found in human LNs [38], suggesting that the complexity of DC subclasses in mice seems to be present in humans as well.

In summary, although the exact mechanisms dictating DC functions are not known, it is likely a dynamic process involving functionally different subtypes engaged in complex interactions with the tissue microenvironment – interactions that can rapidly change in intensity and nature during infection.

1.1.2 ADAPTIVE DEFENSES

Lymphocytes are comprised of T and B cells – the two major components of the adaptive immune system. When the innate immune system is not capable of defeating pathogens on its own, this second line of defense is taken into action.

1.1.2.1 T cells

T cells play important roles in cell-mediated immunity. They are defined by their expression of the T cell receptor (TCR) complex composed by two variable αβ chains non-covalently linked to CD3 (composed of γ-, δ-, ε-, and ζ-subunits) [39]. Differentiation of T cells with their designated TCR occurs in the thymus and generates a pool of T cells that may mount strong responses against foreign antigen but do not respond to self-antigens - a process called central tolerance [40].

It has long been known that the αβ chains of the TCR recognize antigens presented in the context of the right MHC-molecule expressed by APCs (CD4 recognizes class-II and CD8 class-I) . The development of the human leukemia T-cell line, Jurkat, further helped describe the role of the TCR complex (including both the αβ chains and CD3 molecule) in signaling transduction and monitoring T cell fate [41].
Most of the T cell pool in mammals is located at mucosal tissues, in particular in the gut-associated lymphoid tissue [2]. T cells play key roles in protection against mucosal infections [2], which is well illustrated in T-cell deficient patients which exhibit an increased infectious rate [42].

T cells provide support to the immune system in mainly two ways: By providing 'helper' function (Th cells), or through direct killing (cytotoxic T lymphocytes (CTLs)). $CD4^+$ T cells are the main cells that provide helper function. They produce cytokines that can instruct other cells to perform specific functions or produce chemokines that help recruit cells to the tissue to help eliminate pathogens or aid in wound healing and immune-regulation. Cytotoxic T cells on the other hand mediate protection by secreting cytotoxic agents, cytokines and other soluble factors or through cell-surface interactions (Fas-Fas ligand (FasL)), which help eliminate pathogen-infected cells. Nevertheless, we need to keep in mind that many exceptions to the aforementioned functional categorization exist.

During T cell development in the thymus, through successful gene rearrangement, each T cell acquires a TCR with a distinct binding specificity [39]. At this stage, T cells are considered naïve (resting) and continue their migratory journey in their quiescent state until they reach secondary lymphoid tissues (spleen and LNs) where they have the potential to become activated. This requires antigen presentation (TCR/CD3 on T cells bound to peptide-MHC on APCs) and cognate interaction between a number of molecules on the cell-surface of T cells and APCs (CD28-CD80/CD86, CD27-CD70, OX40-OX40L, LFA-1-CD54) leading to actin-mediated membrane reorganization of the cells following formation of the "immunological synapse" [43]. This close interaction between T cells and APCs ultimately induce activation of T cells, transcriptional programing, robust IL-2 secretion and T cell proliferation [43]. However, the APC-T cell interaction in the context of a given cytokine milieu will support the differentiation of different T cell subsets. Thus, efficient T cell activation and differentiation can be simplistically summarized into a three-step model; 1) antigen presentation, 2) co-stimulation, and 3) polarization generated by cytokines in the environment [44].

1.1.2.2 $CD4^+$ T cell lineages

T helper cells ($CD4^+$ T cells) can be further subdivided into different subclasses, such as Th1, Th2, Th9, Th17 and Tregs, among others. In response to intracellular pathogens, APCs produce high levels of interferon (IFN)-α/β and IL-12, which drives the differentiation of Th1 cells. This subset is characterized by their expression of the transcription factor (TF) Tbet [45] and the production of prominent levels of IFNγ and TNFα, cytokines that are indespensable for clearing intracellular pathogens and malignant cells. In contrast, IL-4 skews the differentiation of naïve T cells into the Th2 subtype. These cells typically express the TF GATA-3, produce high levels of IL-4, IL-5 and IL-13 and play key roles in defending the host from extracellular pathogens. In addition, Th2-like cytokines have the ability to promote B cells to produce IgE and IgA antibodies that can circulate into mucosal sites and protect against a second pathogen encounter.

Activation of naïve T cells in a milieu rich in IL-4 and TGFβ triggers the differentiation of a recently described group of T cells, named Th9. As their name implies, they secrete the signature cytokine IL-9 (without IL-4) and express the TF GATA-3 [46]. Studies in murine disease models have suggested Th9 cells to play important roles in tumor protection and in the pathogenesis of diseases, such as allergy, colitis and experimental autoimmune encephalomyelitis [47].

Infection of extracellular pathogens that are not adequately eliminated by Th1 or Th2 cells can be eliminated by a distinct T cell subset, namely Th17 cells. Upon bacterial or fungal infection, large amounts of TGFβ and IL-6 can be produced by innate cells, which together with IL-22 and IL-23 stimulation trigger Th17 differentiation. Their lineage-specific TF RORγT and high production levels of IL-17 helps define their lineage [48]. Although crucial for protection against pathogens, they are potent inducers of tissue inflammation and have lately been associated with the pathogenesis of many human inflammatory conditions [48].

All of the T cell lineages mentioned play specific protective functions against pathogens and are vital for keeping the host healthy. However, uncontrolled activation of any T cell lineage may cause disease. Thus, the immune system needs to tightly control effector T cell responses where Tregs are considered an important checkpoint in this regard.

1.1.2.3 T regulatory cells

T regulatory cells (Tregs) are essential for preventing autoimmune responses by maintaining tolerance to self-antigens. This is well illustrated by their ability to prevent autoimmune diseases like type 1 diabetes [49]. They are also crucial in preventing sustained inflammatory responses and have concurrently been implicated as regulators of chronic inflammatory conditions, like asthma [50].

Forkhead box protein 3 (Foxp3) is the key regulator of Treg function and differentiation [51]. Their crucial role in preventing adverse immune reactions is best illustrated in patients with loss-of-function in the Foxp3 gene, which suffer from severe autoimmune responses, persistent eczema and colitis [52]. This is replicated in the scurfy mouse model which lack $Foxp3^+$ cells and concurrently show similar phenotypes as the human counterpart [53]. Although Tregs comprise a heterogeneous population of cells, including those lacking Foxp3 expression (Tr1 and Th3 cells), the function of these cells *in vivo* is far more elusive. Therefore, in this section I will focus on, and refer to Tregs as $CD4^+$ T cells expressing Foxp3.

Tregs can be divided into two major subsets; Natural Tregs that differentiate in the thymus, and induced Tregs, which develop in the periphery. Development of Tregs can occur in response to low doses of antigen [54], commensals [55], tolerogenic DCs [56] or by DCs in the presence of TGFβ [57]. They are commonly found at mucosal sites, where they are likely involved in inducing tolerance to innocuous antigens, like commensals, food or pollen.

Several suppression mechanisms have been attributed to the function of Tregs. These include their ability to secrete inhibitory cytokines (such as IL-10 and TGFβ) and serine proteases or through the expression of membrane-bound TGFβ [58]. Furthermore, studies suggest that Tregs can mediate suppression by modifying DC biology. In fact, intravital microscopy studies in the LN have shown that interaction between antigen-specific T cells and DCs is reduced in the presence of Tregs [59], whereas the interactions between Tregs and DCs is increased [60].

1.1.2.4 B cells

B cells and their antibodies make up an essential aspect of the immune system and are referred to as the antibody-mediated response or humoral immunity. The developmental stages of the B cells occur in the BM and involves random rearrangements of the DNA that ultimately gives rise to a B cell pool with diverse antibody specificity [61]. Each antibody contains a heavy chain (H-chain) and a light-chain (L-chain) with a conserved and a variable region (the latter responsible for binding to antigens). B cells egress from the BM in an immature state expressing the antibody subtype IgM on their surface, which constitutes the B cell receptor (BCR). Upon reaching secondary lymphoid tissues (spleen and LNs), B cells are considered mature and express both IgM and IgD on their surface and can now undergo activation.

B cell activation can occur through T cell dependent- or independent processes. T cell dependent activation involves binding of a soluble antigen to the specific BCR (clonal selection) leading to its endocytosis. Intracellular processing of the antigen generates fragments that can be re-expressed on the B cell surface and presented to Th2 cells in the context of MHC class II. Alongside, IL-4 receptors are increased on the B cell surface and IL-4 signaling together with additional activation signals ultimately triggers B cell division and antibody class switching into IgG, IgA or IgE. Additional

cytokines produced by Th2 cells (IL-2, IL-5 and IL-6) cause B cells to differentiate into plasma cells able to secrete large amounts of secreted antibodies with the ability to neutralize toxins and promote phagocytosis [61]. Since mucosal immunity depends on antibodies found in external body fluids, such as tears, saliva, intestinal juice and breast milk, approximately 80% of the total plasma cell population is found at mucosal sites [2]. In addition to plasma cells, long-lived memory B cells are also generated which undergo phenotypic changes allowing them to react more efficiently to a second antigen encounter. T cell independent responses on the other hand are mounted by polysaccharide antigens containing multiple repeating epitopes that can cross-link antibodies and thereby activate B cells without the need of T cell help.

The textbook view of B cell generation assumes that B cells are generated in the bone marrow (BM). Although true, Alt *et al* recently broke a paradigm by showing that B cells can also arise in the mouse gut during a short period after birth in response to colonization of the microbiota [62]. This observation is consistent with the evidence showing that also T cell differentiation is regulated by the microflora and together supports the ancient "jaw hypothesis" postulating that the adaptive immune system originated in the gut [63].

1.2 EXTRACELLULAR VESICLES

Multicellular organisms communicate through various mechanisms including cell-cell contact and through secreting soluble proteins and more complex entities, including extracellular vesicles (EVs). These vesicles can travel and reach distant locations where they can interact with cells and affect their biology in various ways. Although initially considered cell garbage, there is increasing evidence supporting their contribution in various biological settings, including cell-to-cell communication, immunity, development, neurobiology and microbiology.

Mammalian cells produce many different types of vesicles, both during physiological settings and during disease. Due to lack of available methodology able to selectively discriminate between subpopulations of EVs and the lack of well-defined markers for specific vesicles, the research community of EVs is currently coping with an imperfect nomenclature. As a consequence, researchers have invented numerous names for EVs reflecting subcellular origin (ectosomes, exosomes, apoptotic bodies, microvesicles), specific function (tolerosomes) or cellular origin (prostasomes). However, a more generic term can be applied and in this section I will refer to exosomes as endosomally-derived vesicles and microvesicles as those generated at the plasma membrane from different cells.

1.2.1 PLASMA MEMBRANE-DERIVED VESICLES

Microvesicles (MVs), often called microparticles, ectosomes or shedding vesicles, can be traced back to 1946 in which researchers were studying the contribution of blood coagulating factors. Chargaff *et al* discovered that platelet-free plasma had potent clotting properties [64] and it was not until 20 years later that Webber and Johnson

found that platelets released small vesicles with pro-coagulant function [65]. Over the past few decades, several studies have confirmed these early findings and further shown that additional cell types can produce MVs, including tumor cells [66]. In fact, elevated levels of MVs with pro-coagulant activity have been found in plasma from patients with cancer, suggesting a contribution of these vesicles in the hyper-coagulant state found in patients [67]. Most of the current research has however focused on the use of MVs as disease biomarkers, whereas limited functional data is available. However, recent elegant work by Choudhuri *et al* showed that the immunological synapse formed between APCs and T cells is indeed a cavity filled with TCR-enriched MVs [68]. This suggests that shedding of TCR through MVs (lacking TCR signaling activity), might constitute a novel mechanism to down-regulate T cell activation.

MVs are phenotypically defined as 100-1,000 nm in diameter. They are generally believed to form at the plasma membrane through a budding mechanism involving loss of phospholipid asymmetry through a calcium-dependent or cytoskeleton-dependent mechanism [69]. Phosphatidylserine (PS) is often exposed on their surface and is thought to mediate their uptake and clearance by macrophages [70]. Notably, there is currently no marker that distinguishes MVs from other types of vesicles, such as exosomes [71]. However, different EVs can be categorized according to several features, including size, density in sucrose, morphology seen by electron microscopy, sedimentation, lipid composition, protein composition and subcellular origin (see Table 1).

Table 1. Comparison of three types of EVs: exosomes, microvesicles and apoptotic vesicles.

Feature	EXOSOMES	MICROVESICLES	APOPTOTIC VESICLES
Size	30-100 nm	100-1,000 nm	50-500 nm
Density in sucrose	1.15-1.19 g/ml	ND	1.16-1.28 g/ml
Appearance by electron microscopy (EM)	Cup shape*	Irregular shape and electron-dense	Heterogeneous
Sedimentation	100,000 g	10,000 g	1,200 g, 10,000 g or 100,000 g
Lipid composition	Cholesterol, sphingomyelin, ceramide and phosphatidylserine	Cholesterol and phosphatidylserine	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, metalloproteinases and selectins	Histones
Intracellular origin	Endosomes	Plasma membrane	ND
Main reference	72-74	75, 76	77, 78

*This is an artificial effect caused by dehydration during processing of vesicles for EM analysis.
ND: not determined. Modified from [79].

1.2.2 ENDOSOMALLY-DERIVED VESICLES

Exosomes, in contrast to MVs, are formed inside the cell within endosomal compartments. They were discovered 30 years ago during an attempt to understand the process involving the loss of the transferrin receptor (TfR) during red blood cell maturation [80]. At this point in time, researchers had long been hypothesizing that the receptor was down-regulated by endocytosis-mediated lysosomal degradation [81]. Surprisingly, in 1983 Harding *et al* found that the TfR was rarely detected in the lysosomal compartments but instead found at the membrane of small vesicles within the lumen of late endosomes [80]. Seen by electron microscopy, it appeared that these vesicles were expelled to the extracellular environment seen by the fusion of the endosomal- and the plasma membrane. A few weeks later, R. Johnstone's group published similar observations [82] and subsequent work by both groups helped establish the novel concept of vesicle-mediated receptor shedding [83, 84]. Since then, major advances have been made in our understanding of the biogenesis and function of exosomes beyond their initially postulated function as mere cellular garbage [73].

1.3 EXOSOMES

1.3.1 ISOLATION AND CHARACTERIZATION OF EXOSOMES

Exosomes can be distinguished from other types of vesicles based on their size (around 30-100 nm in diameter), morphology (cup shape seen by electron-microscopy (EM)), buoyant density (1.15-1.19 g/mL), sedimentation by ultracentrifugation (100,000 x g), and protein composition (enriched in tetraspanins) (see Table 1). The most established and commonly used protocol for isolating exosomes involves sequential ultracentrifugation of cell supernatants or body fluids. However, since it is a tedious and time-consuming method, alternative isolation protocols have been developed, including ultrafiltration, which is less labor intensive and requires no special equipment [85]. In addition, several commercial isolation kits are now available, such as "ExoQuick" from System Bioscience, which offer an "easy" and "quick" method for exosome purification. However, these kits are limited by the lack of specificity for precipitating exosomes in relation to other types of vesicles and cellular debris. As a result, these kits are not suitable when using exosomes for functional studies, but can be accurately applied for the discovery of disease biomarkers. The use of volume-excluding polymers like polyethylene glycol is also used for exosome isolation, although yielding a rather impure isolate, just like the commercial kits. Taken together, ultracentrifugation is therefore the only widely accepted method for exosome purification so far, and when combined with further methods like sucrose density purification or immunoprecipitation, yields an enriched population of exosomes.

Detection of single exosomes is difficult because of their small size. They are impossible to detect with conventional flow cytometry. Therefore, analysis of exosomes by flow cytometry typically requires capture of exosomes on antibody-coated beads that are within a detectable size (typically around 4-4.5 μm ∅). Since each bead has the ability to bind many exosomes, this method has the limitation of

not being able to quantify vesicles or to make distinctions between phenotypically different subpopulations. However, recent methodological advances have been made that enables detection of exosomes at a much higher resolution. For example, by using a special flow cytometer (Becton Dickinson Influx flow) equipped with a high-power laser, it has been feasible to detect fluorescently-labeled vesicles that are around 100 nm [86]. In addition, nanoparticle-tracking analysis (NTA) is capable of measuring the total size distribution of vesicles within a heterogeneous sample (~50 nm to 1 mm) by measuring the Brownian motion of vesicles in liquid [87]. However, several other conventional methods are regularly used to characterize exosome composition of whole exosome purifications, including western blot, enzyme-linked immunosorbent assay (ELISA) and mass spectrometry, just to name a few.

1.3.2 EXOSOME BIOGENESIS

Mature endosomes containing intraluminal vesicles (ILVs) are called multivesicular bodies (MVBs). These organelles have two fates; they can fuse with the lysosome for protein degradation, or back-fuse with the plasma membrane and release the ILVs into the outer milieu as exosomes.

Formation of MVBs partly depends on the ESCRT- (endosomal complex required for transport) machinery which is composed of at least 20 proteins that form four ESCRT complexes ((the ESCRTs -0 (e.g. HRS, STAM1) –I (e.g.TSG101), -II, and –III) and associated proteins (VPS4, VTA1 and ALIX)) [88]. This group of proteins recognizes and targets monoubiquitinylated proteins into the endosomal compartment, promotes membrane deformation, inward budding of the MVB membrane and final scission of the vesicles into the luminal space. Since exosomes derive from ILVs, it has long been assumed that the ESCRT proteins are involved in exosome biogenesis. In addition, the notion that exosomes from various cell types carry ESCRT proteins (ALIX, TSG101) [77, 89], strongly supports an ESCRT-dependent mechanism. Accordingly, several studies have found a role of selected ESCRT components in the formation of exosomes. For instance, a recent study identified ALIX as an important component for exosome formation and sorting of syndecans [90]. In addition, a study targeting 23 proteins of the ESCRT machinery in MHC class II expressing HeLa-CIITA cells showed that several ESCRT-components alter exosome secretion by decreasing (HRS, STAM1, TSG101) or increasing (VPS4) exosome release [88]. Interestingly, in the same study it was found that cells produce a heterogeneous population of exosomes based on size and protein composition that are altered differently by the distinct ESCRT genes. These findings suggest that individual components of the ESCRT machinery have distinct roles in the formation/secretion of specific exosome subpopulations.

Despite the accumulated evidence supporting an ESCRT-dependent mechanism in exosome-formation, ESCRT-independent pathways are also likely involved since alteration of the ESCRT machinery does not completely abrogate the formation of MVBs and ILVs [91]. In fact, it has been established that secretion of exosomes in oligodendrocytes depend on a mechanism involving ceramide-formation [92]. On the other hand, studies in melanoma cells showed that ceramide formation is unnecessary

for MVB- and exosome biogenesis but instead relied on the expression of the tetraspanin CD63 [93]. Furthermore, recent studies support a function of tetraspanins in defining exosomal protein composition by monitoring exosome cargo selection [94]. Other components, such as the tumor suppressor protein p53 and associated transcript TSAP6 have also been assigned a role in exosome formation [95].

The mechanism involved in the release of exosomes from the cell is far from resolved. However, several lines of evidence support a role of different Rab GTPases in this process, partly depending on the cell type. For example, Rab11 is involved in Ca^{+}-induced secretion of exosomes in an erythroleukemia cell line [96], whereas Rab35 operate in oligodendrocytes [97]. More recently, a study targeting 59 different Rab GPTases in HeLa cells found that knock-down of Rab27a or Rab27b lead to a significant reduction in exosome release [98].

In summary, compiled results support the hypothesis that different machineries for exosome formation/secretion operate in different cell types. However, the notion that different MVBs and ILVs [99], as well as different subpopulations of exosomes exist within a cell, suggests that independent- or partially overlapping sorting machineries co-exist within a given cell type.

1.3.3 EXOSOME COMPOSITION

Many methods have been applied to characterize the molecular composition of exosomes, such as proteomics, lipidomics and transcriptomics. These studies have revealed that exosomes do not acquire random components from the originating cell, but are actually equipped with a selection of proteins, lipids, mRNAs and miRNA, suggesting a regulated exosomal sorting mechanism.

Due to their endosomal origin, exosomes contain components of the endosomal compartment (Rab GTPases, SNAREs, Annexins and Flotillin) and the ESCRT-machinery (ALIX, TSG101). In addition, the tetraspanins (e.g. CD9, CD63, CD81 and CD82), which belong to a group of membrane bound proteins found at the plasma- and endosomal membrane, are commonly enriched on exosomes (>100 fold relative to the transferrin receptor) [100]. These proteins gather into microdomains and are often attributed as lipid raft-like, but are however distinct from lipid raft domains [101]. Nevertheless, exosomes carry proteins that are often associated to lipid rafts, including glycosylphosphatidylinositol-anchored proteins and flotillin [102]. Relative to the plasma membrane, the exosomal lipid bilayer is enriched in cholesterol, sphingomyelin, hexocylceramides and phosphatidylserine [74, 103], which may increase their stability *in vivo*.

ExoCarta (http://www.exocarta.org), which is a database of exosomal proteins (as well as lipids and RNAs) have revealed the presence of several exosome-enriched proteins regardless of cell-origin. A few examples are the heat-shock proteins, metabolic enzymes, ATPases and cytoskeletal proteins. However, cell-specific markers can also be detected on exosomes, including A33 (intestinal epithelial-derived) [104],

MHC II and CD86 (APC-derived) [105] and CD3 (T cell-derived) [106]. For a schematic picture of an exosome, see figure 1.

A breakthrough in exosome research occurred when exosomes were found to carry both mRNA (and miRNA) that could be translated into proteins in recipient cells [107]. Later, RNA-containing exosomes were also detected from the supernatant of other cell types [108] and body fluids [109] and found to carry a selected enrichment of RNAs compared to the originating cell. These observations opened up a whole new concept of exosome-mediated intercellular communication involving exchange of genetic material between cells.

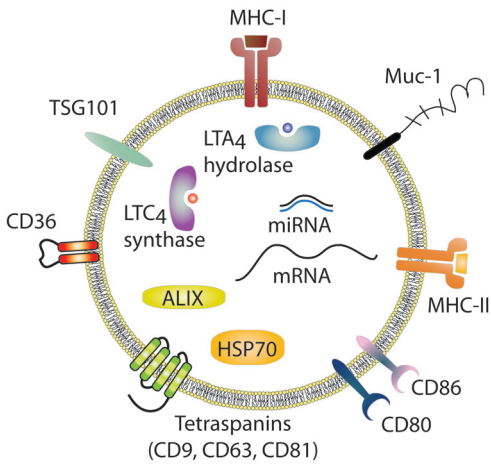

Figure 1. Schematic picture of an exosome, with examples of molecules found in exosomes.

1.3.4 EXOSOMES OF THE INNATE IMMUNE SYSTEM

Virtually all immune-competent cells produce exosomes, including those of the innate- and adaptive arm [79]. Most of the research on functional aspects of exosomes has, however, focused on APC-derived exosomes, in particular DC-exosomes, due to their potent ability to induce antigen-specific immune responses.

1.3.4.1 DC-exosomes

Back in 1998 studies on DC-derived exosomes found that exosomes loaded with tumor antigens could inhibit tumor growth in a manner that was MHC- and $CD8^+$ T cell dependent [110]. These studies laid the foundation for the hypothesis that DC-exosomes could play a role in initiating adaptive immune responses by carrying molecules important for antigen presentation (MHC class I and II, co-stimulatory molecules CD80 and CD86 and adhesion molecule CD54) [111]. Later on, various attempts were made to

scrutinize the mechanism of exosome-dependent T cell activation and it was found that exosomes carrying MHC-peptide complexes can directly bind to the cognate TCR of primed $CD4^+$ T- [105, 112] and $CD8^+$ T cells [113]. However, in order for DC-exosomes to activate naïve T cells, they first need to be captured by a recipient DC, likely due to the requirement of co-stimulatory molecules [114] and cytokine production by DCs, and the need of LFA-1 on (activated) T cells for efficient binding of CD54-bearing exosomes [112]. Once bound to the recipient cell, DCs can directly make use of the MHC-peptide and activate T cells [105] or recycle the peptide onto their own MHC molecule [111]. Hence, exosomes from DCs have been implicated in peptide- or MHC-peptide spreading to increase the number of DCs able to present a given antigen [115].

Most research has been performed on exosomes from *in vitro* propagated bone marrow-derived DCs (mouse) or from human monocyte-derived DCs (MDDCs). Studies have shown that the maturation state of the cell can influence the ability of DC-exosomes to elicit an immune response. For instance, those from mature DCs carry increased levels of MHC class II, CD54 and co-stimulatory molecules and have an enhanced ability to induce T cell priming [116] and antigen-specific IgG responses *in vivo* [117] compared to those from iDC. In contrast, exosomes from iDC can induce tolerance rather than immunogenicity, as exemplified in a mouse cardiac allograft model [118]. DC-exosomes further carry CD1d molecules, which can be loaded with lipid antigens to activate NKT cells, thereby helping to amplify innate and adaptive immune responses [119]. Due to the ability of DC-exosomes to induce antigen-specific immune responses, they are being exploited in the clinic as a promising new cell-free vaccine against tumors [120, 121] and evaluated experimentally as vaccine candidates for other pathologies.

1.3.5 EXOSOMES OF THE ADAPTIVE IMMUNE SYSTEM

1.3.5.1 T cell-exosomes

T cells secrete significantly more exosomes upon activation [122]. Initial studies on activated human T cells performed by a group in Spain in 1999, showed that exosomes produced by these cells carried bioactive FasL with the ability to induce apoptosis in neighboring T cells [123]. These results provided the basis for the novel concept of T cell-exosomes as an autocrine or paracrine mode of immune regulation. Studies have shown that exosomes from ovalbumin (OVA)-specific $CD8^+$ T cells can target OVA-DC and induce apoptosis via Fas-FasL interactions as well as down-regulate peptide/MHC I expression on DCs [124]. Furthermore, exosomes from OVA-specific $CD4^+$T cells have also been shown to bind OVA-DC and inhibit CTL mediated immunity, likely by masking the MHC on DCs [125]. Taken together, compiled evidence support T cell-derived exosomes as regulators of immune responses.

1.3.5.2 B cell-exosomes

As with T cells, B cells also produce more exosomes upon certain activation stimuli, such as in response to antigen-specific $CD4^+$ T cells and CD40/IL-4 signaling [126].

Similar to exosomes from DCs, exosomes from B cells carry antigen presenting molecules and during cognate interaction between B- and $CD4^+$ T cells, B cells release MHC-II carrying exosomes that can activate primed, but not naïve T cells [127]. B cell-derived exosomes have further been implicated as promoters of allergic responses by their ability to present allergens and promote proliferation and cytokine release in allergen-specific T cells [128]. Furthermore, B-cell exosomes from Epstein Barr virus (EBV) infected cells can transfer EBV-proteins to non-infected B cells [129] and miRNA to DCs [130], suggesting exosomes as a novel inter-cellular communication mechanism exploited by virus.

1.3.6 EPITHELIAL-DERIVED EXOSOMES

Exosome release is not confined to the immune system but has been found in a myriad of other cell types, including epithelial [131] and endothelial cells [132] and cells from the nervous system [133].

As mentioned previously, due to the location of epithelial cells at mucosal sites, these cells need to be in constant communication with immune cells located at the basolateral side in order to maintain tissue homeostasis. Hence, exosomes at mucosal sites, including those produced by mucosal epithelial cells, are likely involved in coordinating and exchanging cellular information. It was initially shown that human intestinal epithelial cell lines secrete exosomes at the apical and basolateral side at steady state, and at increased levels during inflammatory conditions [134]. However, immunohistochemistry staining of IECs in human biopsies found only expression of exosome-associated markers (CD63, MHC I and II) at the basolateral side, suggesting a polarized exosome-secretion mechanism [135]. The expression of MHC II on IEC exosomes and the lack of co-stimulatory molecules could suggest a tolerogenic function based on studies showing that liposomes carrying MHC II but lack co-stimulatory molecules cause anergy in $CD4^+$ T cells [136]. In fact, rat IEC-derived exosomes, named tolerosomes, have been shown to induce antigen-specific tolerance when administered intraperitoneally (i.p) in rats [137] and prevent allergic sensitization in a model of allergic asthma [138]. In contrast, studies by Van Niel *et al* found no tolerogenic effect of IEC-derived exosomes carrying OVA, but were instead capable of inducing a strong OVA-specific immune response when administered i.p [139]. Furthermore, studies by Mallegol *et al* showed that exosomes from IECs could transfer and target luminal antigens preferentially to DCs at the basolateral side and lowering the threshold level of antigen presentation required for activation [140]. Therefore, although exosomes released by IECs from the basolateral side seem capable of carrying luminal antigens that may likely interfere with immune cells in the lamina propia, further studies are required to elucidate their immune function and cellular target.

Kesimer *et al* found that also human tracheobronchial epithelial cells release exosomes. These vesicles carried mucins on their surface (MUC1, MUC4 and MUC16) and could bind and neutralize human influenza virus [141], suggesting a potential role for exosomes in mucosal innate defense. More recently, immunohistochemical analysis of

lung tissue revealed that exosome-markes (CD63, CD81 and Rab-5b) were mainly confined to bronchial epithelial cells (BECs) and macrophages, suggesting that BECs in the lungs might be a key player involved in exosome-mediated intercellular communication [142]. Furthermore, under the influence of IL-13, BECs significantly increased exosome secretion and these exosomes were able to induce the proliferation and chemotaxis of monocytes compared to exosomes from untreated cells [142]. Thus, epithelial cells exposed to Th2 polarizing conditions (mimicking an allergic response) seem to produce enhanced levels of exosomes with the ability to increase inflammatory cell proliferation. Therefore, in the case of asthma, IL-13-induced exosome release might potentiate an ongoing inflammatory response.

1.3.7 EXOSOMES IN HEALTH AND DISEASE

Up to date, exosomes have mainly been purified from *in vitro* cell cultures and studied *in vitro* and *in vivo* in their ability to influence disease mechanism. These studies have revealed that exosomes can trigger, promote or even alleviate disease phenotype in a context dependent manner. Nevertheless, exosomes likely have important roles beyond pathologies due to detectable levels of these vesicles in body fluids of healthy individuals.

1.3.7.1 Body fluid exosomes in health

The lack of proper methodological tools, such as exosome KO mice and exosome-specific markers, has put limitations on our understanding on the role of exosomes *in vivo*. Nevertheless, the presence of exosomes in all human body fluids analyzed so far, including urine [143], saliva [144], breast milk [145], plasma [146], and malignant effusions [147], suggests an important function of exosomes *in vivo*. Furthermore, the presence of detectable levels of exosomes in several body fluids at steady state suggests a role of exosomes in maintaining the body healthy. Accordingly, studies on exosomes from seminal fluid have shown to down-modulate NK function, which is thought to prevent immune-mediated sperm destruction [148]. Furthermore, exosomes from the placenta have been reported to carry FasL and can be detected in serum during pregnancy at higher levels in women delivering at term versus women delivering pre-term [149]. Since FasL is involved in providing clonal deletion of lymphoid cells, these results suggest that placenta-derived exosomes could help protect the fetus from immune-recognition and destruction. In fact, cultures of human placental explants were shown to release exosomes carrying FasL and TRAIL, which were able to induce apoptosis in T cells and activated PBMCs of healthy individuals [150]. Furthermore, Hedlund *et al* further reported the presence of NKG2D ligands on placenta-derived exosomes with the ability to down-modulate NKG2D-dependent killing of PBMCs *in vitro* [151]. Taken together, exosomes produced by the placenta could have a role in maintaining a state of immune-privilege during pregnancy in order to avoid rejection of the fetus. Blood-born exosomes have also been implicated as immune-regulators with the ability to suppress antigen-specific inflammation in a Fas-FasL dependent-manner [152] or induce antigen specific tolerance in a mouse model of allergic asthma [138]. Thus, exosomes in

blood might be involved in dampening antigen-specific immune responses and could play a role in preventing autoimmunity and/or allergic responses.
In summary, these results indicate that exosomes present in body fluids during steady state may act as regulators of immune responses.

1.3.7.2 Exosomes in disease

Exosome research has greatly focused on the role of tumor-derived exosomes in mediating disease and as potential diagnostic biomarkers. Accumulating functional data have established a role of tumor-derived exosomes as a mechanism of immune-escape by inhibiting NK- and T cell function [153], blocking DC maturation [154], inducing myeloid-suppressor cells [155], or enhancing Treg activity [153]. Furthermore, melanoma-derived exosomes have been shown to regulate tumor growth and metastasis *in vivo* by transferring the oncogenic protein MET to bone-marrow progenitor cells [156]. The increase in levels of exosomes in plasma from cancer patients [157, 158] further supports a role of exosomes in tumor pathogenesis.

Exosomes have also been linked to the spread of proteins involved in neurodegenerative disorders and autoimmune diseases. For instance, the amyloid-β peptide involved in the pathogenesis of Alzheimer's disease (AD) can be secreted within exosomes [159]. Notably, accumulation of exosome-associated proteins within plaques of AD patients further reinforces a role of exosomes in AD pathogenesis. Similarly, α-synuclein, which is central in the pathogenesis of Parkinson's disease, can be released by cells through exosomes [160], indicating a potential role of exosomes in accelerating disease. Likewise, exosomes isolated from the synovial fluid of individuals with rheumatoid arthritis (RA) carry autoantigens (citrullinated proteins) involved in disease pathogenesis [161]. In addition, exosomes released by fibroblasts obtained from the synovial fluid of RA patients carry membrane-bound TNFα which exhibit cytotoxic functions and can render T cells resistant to apoptosis [162].

1.3.8 EXOSOMES AND PATHOGENS

Since most data in the area of exosomal immunology have focused on their role in cancer, relatively limited data is available on their role in microbial pathogenesis. It has been reported that single cell eukaryotic pathogens including the fungi *Cryptococcus neoformance* [163] and *Malassezia sympodialis* [164], and the protozoan *Leishmania major* [165] secrete vesicles that carry virulence factors thought to help spread disease. Furthermore, cells infected by intracellular pathogens (e.g. bacteria, fungus and parasites) can also secrete exosomes carrying PAMPs, which can limit or spread infection, likely depending on the type of pathogen and the target cell [166]. For instance, exosomes from peripheral blood of mice infected with malaria (*Plasmodium yoelii*) carry parasite proteins, which upon transfer to naïve mice can promote survival and clearance of the parasite upon infection [167]. On the other hand, DCs infected with the yeast *M. sympodialis* can release nanovesicles that can trigger IL-4 production in autologous PBMCs of atopic eczema patients to a higher extent than in healthy individuals [164].

Exosomes have further been implicated in virus infection. The 'Trojan horse' hypothesis has been postulated for retroviruses, such as HIV, and suggests that virus make use of the exosomal-biogenesis machinery in order to avoid immune recognition and increase dissemination [168]. For instance, Herpes simplex virus-1 envelope glycoprotein B has been shown to deviate HLA-DR into the exosomal pathway and therefore circumvent its target to the cell-surface [169]. Furthermore, HIV has been reported to incorporate the viral protein Nef to exosomes, thus triggering apoptosis in bystander T cells [170]. Finally, EBV has been shown to encapsulate EBV-specific miRNA into exosomes with potential repression effects on target genes in recipient- non-infected cells [130].

1.3.9 EXOSOMES IN THERAPY

The ability of exosomes to shape immune responses has also lead to their use in immunotherapy, particularly as a vaccine treatment against cancer. So far, two phase I clinical trials have included exosomes from iDCs loaded with tumor antigens as vaccine treatment in patients with melanoma and non small cell lung cancer [120, 121]. Although exosomes were well tolerated in patients, they were shown to have limited therapeutic benefits. Thus, efforts are made to improve immunogenicity of exosome tumor vaccines [119, 171].

Since exosomes contain proteins and genetic material that can be delivered to target cells, they are considered well suited as vehicles for drug delivery and gene therapy. In addition, the advantage of using such vesicles, as opposed to other delivery vectors is that they are biocompatible, immunologically inert, can be patient-derived and have the ability to cross the blood-brain-barrier [172]. In fact, exosomes loaded with siRNA carrying a brain-specific peptide on their surface were able to deliver strong RNAi responses specifically to cells in the brain of mice [173]. In addition, exosomes from human plasma have been shown to deliver siRNA to monocytes and lymphocytes and mediate efficient RNAi responses [174]. Current studies are also exploiting exosomes as potential gene therapeutic vehicles in cancer. One proposed strategy involves the loading of exosomes with exogenous miRNA with the aim to repress translation of pathology-related genes in tumor cells [175].

Due to the involvement of exosomes in the pathogenesis of several diseases they could potentially be used in the future as therapeutic targets by: *i*) interfering with exosomal release (e.g. blocking ceramide production, certain Rab GTP-ases, or interfering with syntenin-syndecan interactions), *ii*) inhibiting exosome-uptake or *iii*) blocking pathogen-promoting components on exosomes. However, although exosome biology is considered a promising new therapeutic research avenue, it is still at an emerging state, and further knowledge on exosome biogenesis, function and mode of cell-interaction is required for their translation into the clinic.

1.4 MUCOSAL IMMUNOLOGY

Organs, like lung, gut and mammary gland have evolved mechanisms to respond adequately to both dangerous and non-dangerous triggers. However, these mechanisms sometimes fail to respond appropriately, leading to inflammation and disease development, such as pulmonary sarcoidosis, food allergy, asthma or mastitis.

1.4.1 AIRWAYS

1.4.1.1 General anatomy

The respiratory tract can be divided into two sections: the upper (including nose and nasal cavity) and the lower compartments (trachea dividing into bronchi, bronchioles and alveoli). The human lung can make room for 5 L of air with its surface over 120 m^2 [176], which is comparable to the surface area of a medium-sized house. This is due to the presence of millions of alveoli (~480 million) located at the end of the conducting airways, each having a diameter of approximately 0.2 mm [176]. The upper respiratory tract and the trachea are lined with mucus secreting goblet cells and ciliated epithelium that trap antigens and clear it away by their rhythmic beat. By contrast, a thin, non-ciliated Type I and II epithelium surround the alveoli to allow efficient diffusion of gases. However, air does not only contain gases, but carries also pollution, fungal spores, bacteria, virus and allergens at various amounts depending on the environment. Therefore, efficient antigen handling and removal is key to maintain safety in the lungs.

1.4.1.2 Antigen uptake in the lung

Despite the enormous antigen exposure in the lungs through breathing air, adverse pulmonary immune responses are relatively rare. This is due to the tightly balanced immune mechanisms that govern the lungs and that function to direct immune effector responses towards harmful pathogens, while inducing tolerance against innocuous microbes or particles. In this regard, proper antigen processing and handling by lung APCs likely play important roles in maintaining lung homeostasis.

A large body of literature have identified lung DCs and alveolar macrophages (AM) as the main cells responsible for antigen uptake [177]. However, in contrast to AM, DCs are the major cell type responsible for carrying antigens to the draining LNs and inducing T cell responses [178]. Three subsets of DCs can be found in the naïve lung; a small number of pDC and two migratory subtypes, $CD103^+$ and $CD11b^{hi}$ [37]. Although both migratory subtypes have shown to be stimulatory rather than tolerogenic [179, 180], they have been proposed to have functionally distinct roles. For instance, studies by Rio *et al* showed that $CD103^+$ DCs nearly exclusively promote $CD8^+$ T cell activation whereas $CD11b^{hi}$ DCs induce proliferation of $CD4^+$ T cells [181]. In contrast, AM which comprise ~95% of the immune cells in bronchoalveolar lavage (BAL), are considered poor antigen presenting cells and non-migratory. Nevertheless, they have recently been suggested to play key roles in tolerance induction by promoting the development of

Tregs [182]. Furthermore, alveolar epithelial cells (AEC) have also been shown to function as APCs [183]. During inflammation, AEC Type II up-regulate MHC class II on their surface and TGFβ secretion, leading to increased conversion of T cells into antigen-specific $CD4^{+}Foxp3^{+}$ Tregs.
How DCs that mainly reside in the tissue during steady-state get access to luminal antigens is a matter of debate. Tracheal preparations have revealed DCs protruding their dendrites into the airway lumen [184]. Further studies supports this notion by the observation that lung DCs are able to capture fluorescently labeled antigens that are too large to pass the epithelium [185]. Taken together, these studies suggest that at least tracheal DCs possess mechanisms to reach across the airway lumen. However, the presence of these protrusions during steady state and their relevance to the whole lung is still elusive. Recent studies trying to address this question reached unexpected results. Using a technique allowing to image living tissue, Thornton *et al* found that during steady state, DCs mainly protrude dendrites across the epithelial barrier in the alveoli [178]. Taken together, it is tempting to speculate that during steady state, a functional epithelial barrier together with AM are involved in promoting tolerance to harmless antigens. However, potentially harmful pathogens that manage to override this line of defense can be picked up by DCs in the lower airways and induce T-effector immunity.

1.4.1.3 Respiratory inflammation

In spite of the many regulatory mechanisms in the lung aiming to prevent pathologies, it occurs that the system fails to sustain a healthy relationship between the antigenic burden and the tissue, thus triggering pulmonary inflammation. Different inflammatory lung diseases (e.g. asthma, COPD and sarcoidosis) display distinct inflammatory mediators and cellular infiltration profiles. However, characteristic features of inflammation can be found in most inflammatory lung diseases.

Inflammation is initiated in response to danger signals (PAMPs or DAMPs) that trigger innate responses, often involving airway epithelial cells and/or airway macrophages. These cells express PRRs that sense and responds to pathogens by producing inflammatory mediators, such as oxygen species, pro-inflammatory cytokines and lipid mediators [186, 187]. These inflammatory factors can directly affect the inflammatory response. Alternatively, they can indirectly support the response by facilitating recruitment of inflammatory cells from the blood to the tissue by up-regulating adhesion molecules (e.g. VCAM-1, E- and P-selectin) on endothelial cells, which bind and arrest cells to the capillary wall. Chemokines produced by lung epithelial cells (e.g. RANTES [188], CCL20 [189], MCP-1 and IL-8 [190]), further helps direct the migration of cells egressing from the blood to the tissue. Upon infiltration, cells can aid in the inflammatory response by producing additional chemokines, driving additional recruitment of inflammatory cells to the site of inflammation, and by producing pro-inflammatory mediators that can stimulate cell activation and tissue damage [191].
A polarized Th1-skewed response is characteristic following lung infection by intracellular bacteria and by virus, whereas Th2-polarized immunity predominates in response to parasites or allergic responses, such as allergic asthma.

1.4.1.4 Etiology of sarcoidosis

Sarcoidosis is a disease that often manifests in granuloma formation of the affected organ, typically the lungs (90% of patients) [192]. The etiology of sarcoidosis is currently unknown. However, several environmental triggers have been (modestly) associated with sarcoidosis, including agricultural employment, mold or mildew, musty odors at work and pesticides [193]. In contrast, environmental factors like animal dust, feather- or down pillows and smoking [194] have surprisingly been negatively associated with sarcoidosis [195]. Nevertheless, no single cause of sarcoidosis has been identified as of yet.

Extensive work supports *Mycobacterium tuberculosis* (MTB) infection as a strong candidate etiological agent for sarcoidosis [196]. Indeed, mycobacterial DNA has been extracted from sarcoidosis tissues in a significant portion of patients [197]. Furthermore, Th1-mediated immune responses against several mycobacterial antigens, including catalase-peroxidase (mKatG), have been described in patients [198]. In addition, patients with tuberculosis (TB), a disease caused by *M. tuberculosis* infection, show remarkable clinical and radiological similarities to sarcoidosis. For instance, granuloma formation is a hallmark of both TB and sarcoidosis that exhibit subtle differences (necrotizing cells in the center of the granuloma in TB while usually absent in sarcoidosis) [199].

In support of the view of sarcoidosis as an infectious disease, microbiological studies have shown that transplantation of sarcoidosis tissue cause granuloma formation in transplanted mice and following organ transplantation in humans [200]. Furthermore, injecting sarcoidosis tissue into the skin of patients, called the Kveim test, results in granulomatous inflammation in sarcoidosis positive patients. This clinical examination used to be a standard diagnostic procedure of sarcoidosis. However, since sarcoidosis might be transmissible, it is rarely used in the clinic nowadays.

Up to 35 % of sarcoidosis patients have Löfgren's syndrome - an acute form of sarcoidosis with favorable prognosis often leading to total remission. Löfgren's syndrome is more common in European populations, in particular Scandinavians, suggesting a strong genetic influence. In fact, genetic predisposition to the disease exists and is highly associated with the HLA-class II molecule, in particular the *DRB1* alleles [201]. For instance, *HLA-DRB1*01* and *HLA-DRB1*04* are thought to protect, whereas *DRB1*03, DRB1*011, DRB1*012, DRB1*14* and *DRB1*15* represent risk factors for sarcoidosis. Furthermore, *HLA-DRB1*03* positive patients with Löfgren's syndrome run a very favorable disease course, while *DRB1*03* negative patients have a more protracted disease that requires careful follow-up [201].

In summary, sarcoidosis is currently considered a heterogeneous disease likely triggered by multiple environmental factors that when combined with genetic predisposition can culminate in disease.

1.4.1.5 Immunology of sarcoidosis

The pathophysiology of pulmonary sarcoidosis likely involves an aberrant immune response against different antigenic triggers in genetically predisposed individuals [201]. Common pathological features can be found in patients, such as non-caseating granuloma formation in the lungs with associated inflammation. In spite of this local inflammatory response, sarcoidosis patients often simultaneously exhibit a paradoxical state of cutaneous anergy to skin test antigens [202].

In an attempt to describe the paradoxical immunity of sarcoidosis, Mathew *et al* found that the peripheral anergic state in patients was associated with a diminished DC function [202]. They speculated that the lower ability of DCs from patients to stimulate T cells could be due to an abnormal antigen uptake or presentation. Another plausible explanation for a reduced peripheral immunity is the marked increase in $CD4^+$ Tregs in the periphery of sarcoid granulomas, in BALF and in peripheral blood of patients with active disease. [203]. These cells showed a potent ability to suppress T cell proliferation but could, however, not completely inhibit TNFα secretion, which may account for their inability to control granuloma formation.

Pulmonary sarcoidosis is often characterized by increased $CD3^+CD4^+$ T cells in BALF with an increase in the $CD4^+/CD8^+$ T cell ratio [204] with activation markers being overexpressed in T cells from BALF compared to blood [205]. These activated T cells produce high levels of Th1-cytokines like IL-2, TNFα and IFNγ, whereas IL-4^+ T cells are hardly detectable in sarcoidosis BALF [206]. NKT cells, which are known to be important in suppressing Th1-biased immune responses [207], have further been found at reduced levels in blood and BALF of patients [208].
Granuloma formation is composed by a core of epithelioid-cells (activated macrophages resembling epithelial cells) and multinucleated giant cells (fused macrophages) surrounded by a ring of B cells, mast cells and predominantly $CD4^+$ and occasional $CD8^+$ T cells. These cells primarily secrete Th1-biased cytokines including IL-2, TNFα and IFNγ, which help sustain granuloma formation and attract additional inflammatory cells [209].

Most individuals with sarcoidosis have full remission of disease without signs of tissue damage. However, in a portion of patients, the disease evolves into a chronic state that can progress into lung fibrosis leading to morbidity [210]. Accordingly, it has been postulated that disequilibrium between effector and regulatory responses might exist which may drive the transmission from acute to chronic disease. This is supported by the observation that during acute disease (often following remission), Tregs from affected lesions dampen certain T cell inflammatory responses [203], whereas Tregs in chronic stages seem incompetent in this regard [211]. However, further studies are required to fully understand the immunological steps leading to severe stages of disease.

1.4.1.6 Asthma definitions

Similar to sarcoidosis, asthma comprises a heterogeneous group of conditions likely reflecting the diverse genetic and environmental factors that underline asthma pathogenesis. Early definitions of asthma involved two phenotypes: extrinsic asthma (often developed during childhood and associated with production of IgE antibodies) or instrinsic asthma (later onset and not dependent on allergic sensitization). These definitions are now considered biased and do not capture the diversity of asthma phenotypes. Therefore, researchers have developed unbiased approaches, including different statistical analysis methods, in order to better define asthma characteristics. A study conducted in 2009 made major advances in this regard. Based on molecular phenotyping of bronchial biopsies from a group of patients with mild-to-moderate asthma, Woodruff *et al* dichotomized patients into a "Th2 high" and a "Th2 low" subpopulation. The "Th2-high" asthma phenotype exhibited higher levels of IL-13 and IL-5 mRNA in the tissue compared to "Th2-low" patients, greater numbers of eosinophils and mast cells in BALF and allergen-specific IgE in blood serum. Therefore, these studies support the novel definition of asthma based on degree of Th2-driven inflammation and further suggests that alternative, non-Th2 driven mechanisms, might be more prominent in a subgroup of patients (Th2-low).

1.4.1.7 Innate immune responses in asthma

Similar to other lung inflammatory diseases, asthma pathogenesis often starts at the airway epithelium. In addition to its barrier function, the epithelium can sense various insults, it being allergens, pathogens or cigarette smoke and respond by producing a series of inflammatory mediators and antimicrobial peptides [212]. It has been shown that injured epithelium releases IL-25, IL-33 and thymic stromal lymphopoietin, which in turn can activate NKT cells, mast cells, eosinophils, basophils and innate lymphoid cells 2 (ILC2) [213, 214]. The ILC2 subclass is able to produce high levels of Th2 cytokines (IL-13 and IL-5) in response to epithelial damage. Furthermore, they also respond with a similar cytokine production profile upon sensing IL-33 produced by virus infected alveolar macrophages [215]. This provides a previously unappreciated link between allergen-induced and virus-induced Th2-drive immune responses. Ultimately, it is believed that a damaged epithelial barrier could more readily provide the entry of otherwise innocuous antigens. Thus, entry of such antigens into a Th2-rich cytokine environment supported by the innate immune compartment, could drive the differentiation of Th2 cells and subsequent production of IgE antibodies [214].

1.4.1.8 Lipid mediators in asthma

Leukotrienes (LTs) are a group of potent pro-inflammatory lipid mediators involved in normal host-defense and in the pathogenesis of various inflammatory diseases, including asthma [216]. They are rapidly produced by eosinophils, basophils, mast cells and alveolar macrophages upon activation at the site of inflammation [217]. For a schematic image of the 5-lipoxygenase (5-LO) synthetic pathway, see figure 2. The synthesis of LTs involves an enzymatic cascade initiated by the cytosolic enzyme

phospholipase$_2$ (cPLA$_2$), which releases arachidonic acid (AA) from the nuclear membrane [218]. The permanently membrane bound protein FLAP (5-LO activating protein) is believed to transfer AA to activated 5-lipoxygenase (5-LO), which then catalyses the formation of 5-hydroperoxy eicosatetraenoic acid (5HPETE) and further conversion to the unstable epoxide leukotriene A$_4$ (LTA$_4$) [219]. In neutrophils and macrophages, this occurs at the nuclear membrane. Two different enzymes can then rapidly metabolize LTA$_4$: the cytosolic enzyme LTA$_4$ hydrolase (LTA$_4$H), which converts LTA$_4$ into LTB$_4$ (ligand of receptors BLT1 and BLT2), and the nuclear membrane enzyme LTC$_4$ Synthase (LTC$_4$S), which conjugates reduced glutathione to LTA$_4$ forming LTC$_4$. The lipid mediator LTC$_4$, and its metabolic products LTD$_4$ and LTE$_4$, are called the cysteinyl leukotrienes (cysLTs) and bind to receptors CysLT 1 and 2 [216].

CysLTs are potent bronchoconstrictors in humans and have therefore been of special interest in asthma research. Additional biological effects of cysLTs include increased blood vessel permeability, increased mucus secretion, airway remodeling and eosinophilic chemotaxis [219]. LTB$_4$ is a potent chemotactic factor, mainly attracting neutrophils, mast cells and monocytes and it has been related to several lung-associated inflammatory diseases [220]. Three receptor antagonists targeting CysLT1 have been developed and are commercially available (pranlukast, montelukast and zafirlukast) [217], while CysLT2 and BLT1/2 receptor antagonists are still under investigation.

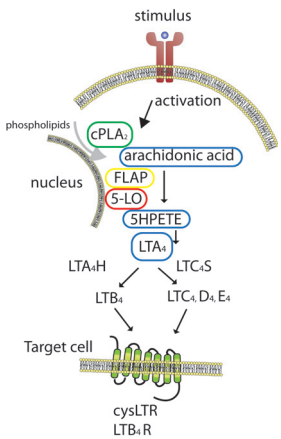

Figure 2. The 5-lipoxygenase (5-LO) pathway of arachidonic acid. Redrawn from [221].

1.4.1.9 Adaptive immune responses in asthma

The prevailing paradigm of asthma pathology involve; a Th2-driven immune response with elevated production of IL-4, IL-5 and IL-13 leading to the recruitment and activation of inflammatory cells to the lungs. This generates mucus hypersecretion, pulmonary inflammation, B-cell isotype switching, and airway hyperresponsiveness. However, T-cell derived adaptive immune responses in asthma is not limited to the Th2 linage. This is particularly true in a subgroup of patients where therapeutic strategies to suppress Th2-responses have proven ineffective in clinical trials [222].

More recently, Th17, Th9 and Tregs have been considered important players in asthma pathogenesis. It has been hypothesized that the increased levels of Th2 cells in the lungs of asthmatics could partially be due to disequilibrium between effector and regulatory mechanism. This is supported by studies showing that Tregs from atopic individuals have reduced suppression function and in some cases the absolute numbers of Tregs are reduced [223].
Emerging evidence suggests that Th17 cells might also contribute to the development of asthma, at least in a subgroup of non-atopic patients having severe airway neutrophilia. This is due to the known role of IL-17 on neutrophil recruitment [224]. However, studies using animal models of allergic inflammation has shown that IL-23, another Th17-derived cytokine, is a potent inducer of neutrophilic and eosinophilic airway inflammation [225]. Nevertheless, it is unclear how far the findings made in allergen challenged animal models can be extrapolated to human disease since they don't reproduce all features of human asthma.

In conclusion, although major advances have been made to understand the complexity of asthma pathogenesis, more studies are required to better categorize different types of asthma with the hope to generate future targeted therapies.

1.4.1.10 Bronchoalveolar lavage fluid-derived exosomes

Bronchoalveolar lavage-fluid (BALF)-derived exosomes from healthy individuals carry antigen-presenting- (MHC I and II) and co-stimulatory molecules (CD80 and CD86) [226], suggesting a potential role of exosomes in local immune responses. Studies by Prado *et al* showed that BALF exosomes from allergen-tolerized mice could confer protection against allergen-induced inflammation in recipient mice [227]. In contrary, preliminary results by the same group imply that BALF exosomes from allergen-sensitized mice can instead aggravate an allergen specific response (N. Prado, C. Thery, M. Villalba, R. Rodriguez, and E. Batanero, unpublished results) [227]. Furthermore, follow-up studies showed that pre-treatment with BALF exosomes from tolerized mice also provided protection against subsequent sensitization with an unrelated allergen, an effect described as "bystander suppression" [228]. Together, these studies suggest that exosomal function varies with the physiological state of the lung. Furthermore, it also sheds light on the potential use of "tolerogenic" exosomes as prophylactic or curative treatment against allergies.

A study in mice recently shed some light on the cellular source of BALF exosomes. The study group reported an increase in exosome production in BALF upon allergic sensitization and data suggest that epithelial cells and macrophages are the main cellular source for exosome-production [142]. In addition, exosomes released by bronchial epithelial cells (BEC) during an inflammatory condition (IL-13), but not from naïve cells, were able to promote monocyte proliferation, suggesting a pro-inflammatory function of BEC-derived exosomes during pulmonary inflammation. BALF exosomes have also been implicated in immune responses to intracellular pathogens. Accordingly, BALF-exosomes isolated from *Mycobacterium bovis* infected mice carried bacterial antigens and could stimulate TNFα production in naïve macrophages [229].

Despite the importance of exosomes in inter-cellular communication, limited data is available on BALF-exosome function and characterization in humans. During the work with this thesis we reported the presence of miRNA in BALF-derived exosomes from both healthy and asthmatic patients [230]. Remarkably, there were significant difference in the miRNA profile between the control group and patients with unprovoked, stable asthma, suggesting a potential role of BALF-exosomes in the asthma-associated inflammatory response.

In conclusion, studies suggest that BALF exosomes might be involved in mediating local immune reactions. However, the type of immune response elicited by BALF exosomes is likely context-dependent.

1.4.2 MAMMARY GLAND

1.4.2.1 Lactation

The mammary gland is a unique organ in mammals with the specific function to produce and secrete milk to the offspring. Therefore, it is not until the female becomes pregnant that the mammary gland reaches full maturation, causing extreme modifications of the gland in order to transform into a milk-secreting organ. Similar to the lower airways, the lactating mammary gland is composed of a branching network of alveoli that form hollow cavities surrounded by a single epithelial cell layer able to secrete milk. Myoepithelial cells positioned underneath the milk-secreting epithelium can respond to oxytocin hormone and contract, thus helping to eject the milk from the alveolar lumen [231].

Milk-secreting epithelial cells produce milk through five distinct secretory pathways (see figure 3) [231]. The exocytic pathway (pathway I) mediates the secretion of aqueous solutes (e.g. milk proteins, lactose, oligosaccharides and calcium) while the milk-fat globule (MFG) pathway (pathway II) provides the secretion of lipids by forming lipid-enclosed membrane vesicles (the MFG). The transcytosis pathway (pathway III) promotes endocytosis of exogenous material at the basolateral side of the epithelium, such as secreted IgA (sIgA), leading to the formation of mature endosomes, which can fuse with lysosomes for protein degradation or fuse with the apical side of the plasma

membrane for exocytosis. The membrane transcellular pathway (pathway IV) involves the direct fusion of small molecules (glucose and amino acids) and water across basal and apical plasma membranes. Finally, a paracellular route exists (pathway V) but is only available during pregnancy or during mastitis (breast inflammation). During these conditions, low molecular weight molecules from serum and interstitial fluid can enter through this route.

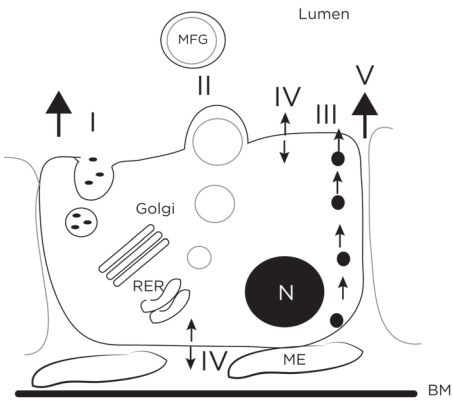

Figure 3. Milk secretory pathways (I-V) by lactating alveolar epithelial cells. I) The exocytic pathway, II) milk fat globule forming pathway, III) the transcytosis pathway, IV) the membrane transport pathway and V) the paracellular pathway. MFG: milk fat globule, ME: myoepithelial cells, N: nucleus, RER: rough endoplasmic reticulum, BM: basement membrane. Redrawn from [231].

1.4.2.2 Breast milk

Breast milk has long been known to contain all necessary nutrients for the development of the newborn. The concept that milk also carries immune-modulatory components has also been acknowledged for quite some time. In fact, studies performed by Paul Ehrlich back in 1892 showed that immunity against plant toxins was transferred to the fetus *in utero* and via breast milk [232]. The protective substance was later shown to be due to antibodies present in milk. Ehrlich referred to this effect as "passive immunity" and was rewarded the Nobel Prize in 1908 for his findings, which helped to pave the way for modern immunology.

Human breast milk is enriched in sIgA, followed by IgM and IgG. These antibodies provides passive immunity to the child that at birth is immunologically naïve and deprived of its own IgA production until almost 30 days after birth [233]. Antigen-specific IgA can be transported to the milk from the blood or produced by IgA^+ B cells homed to the mammary gland from the intestine or lungs through a CCL28-dependent mechanism [234]. Ultimately, sIgA transferred to the neonate can provide protection against intestinal and respiratory pathogens by preventing their adherence to the newborn gut [235].

Human breast milk also contains growth factors (e.g. insulin-growth factor and epidermal growth factor) likely having beneficial effects on both the mammary gland

and the gastrointestinal tract/immune system of the child [236]. Cytokines like IL-1, TNFα, IL-6, TGFβ and IL-10 are present in milk and several cytokines can be induced *in vitro* in T cells and macrophages isolated from milk. Macrophages are the most abundant cell type in colostrum (early milk secreted 3-6 days after delivery) (40-50%) followed by neutrophils (40-50%) and lymphocytes (5-10%) [237]. Many studies have shown that milk leukocytes are taken up by the newborn [238-240], suggesting that they confer active immunity to the child. Other components with immune protective factors in milk are fatty acids, lactoferrin, lysozyme, oligosaccharides and antioxidants. Lastly, recent work has shown that human breast milk also provides commensal and probiotic bacteria to the infant gut with potential effects on the development and maturation of the child's immune system [241].

1.4.2.3 Breastfeeding and allergy

It is well documented that breastfeeding protects the child against diarrheal diseases and is currently the most effective intervention against mortality of children under 5 years of age [242]. However, the relationship between breastfeeding and IgE-mediated allergic disease remains controversial and has been under intense debate for a long time. I will here outline a few of the many reports showing evidence for and against a protective effect of breastfeeding against development of allergy.

A large longitudinal Swedish birth cohort study (BAMSE) found that exclusively breastfed children (≥4 months) had a reduced risk of asthma and atopic dermatitis at the age of 2 [243]. However, this study defined allergies only based on symptoms and not IgE-sensitization. Follow up studies by Kull *et al* found that breast-feeding for ≥4 months protects against asthma [238] and eczema [239] also at 4 years of age, irrespective of IgE sensitization to common allergens. A large Australian longitudinal study (Tasmanian Asthma Study) following patients from the age of 7 to the age of 44 years, showed that exclusive breastfeeding for the first 3 months of children with a maternal history of atopy led to a reduced risk to develop asthma before the age of 7 years [244]. However, they found an increased likelihood to develop asthma after the age of 7. This study did however not take into account IgE-sensitization. Conversely, in a German cohort it was reported that exclusive breastfeeding (≥5 months) in high-risk infants, increased the risk of sensitization to egg white and the children were more commonly diagnosed with eczema at 1 year of age (but not at 2 years) [245]. Lastly, a New Zealand birth cohort following children from age 3 and assessed every 2-5 years from age 9 to 26 years showed that breast-fed children were more commonly sensitized to common allergens compared to non-breastfed children at all ages analyzed [246]. Of note, the study assessed breastfeeding habits retrospectively at age 3 years, with a potential increase in errors due to confounding factors.

A major reason to the controversy in the literature is attributed to flaws and differences in experimental layout. This was illustrated in a multidisciplinary review revising the literature regarding breastfeeding and allergies between 1966 and 2001, where only 56 out of 4323 studies were considered conclusive [247]. One major limitation in the methodology of these studies is the inevitable natural bias due to the incapacity

to randomize infants to a non-breastfeeding control group and the inability to perform blinded breastfeeding. Another important bias is the inclination of mothers with allergic disease to breastfeed longer due to the known health benefits of breast milk. However, other important factors, such as family history of allergies, duration and exclusiveness of breast-feeding, socio-economic status, lifestyle [248], smoking, country of origin etc., are likely to play a role as well [249] and should be controlled for when possible.

1.4.2.4 Breast milk-derived exosomes

Our current knowledge on the function of breast milk exosomes is scarce. In 2007, Admyre *et al* in our group reported the presence of exosomes in human breast milk and showed their ability to induce regulatory $Foxp3^{+}CD4^{+}$ T cells in PBMCs [145]. A following study by us further reported the presence of RNA in human milk exosomes that could be transferred to macrophages, supporting the notion that milk-derived exosomal RNA can be shuttled between cells [109].

Additional studies have mainly focused on analyzing RNA content in milk exosomes. Data by Zhou *et al* show that exosomes in human milk are enriched in immune-relevant miRNAs (30b, 182, 200a and 148a) [250] with the most abundant being miRNA 148a, also highly enriched in bovine milk [251]. These miRNA are resistant to degradation when subjected to multiple freeze and thaw cycles, prolonged incubation in room temperature [251], to RNAse and acidic conditions [252], which can be explained by the encapsulation of miRNA within vesicles. Recently, an interesting study was able to detect exogenous plant miRNA from food in the sera and tissue of several animals, suggesting their durability in the stomach and ability to reach systemic circulation [253]. Thus, it is tempting to speculate that breast milk-derived exosomes carrying proteins and genetic material could be transferred to the child and affect the newborn's immune system.

2 AIMS

Exosomes are nano-sized vesicles capable of modulating immune responses by orchestrating communication between cells. Exosomes isolated from body fluids are by far the most physiological population of exosomes to study. Therefore, research on these exosomes is more likely to yield *in vivo* relevant results, as opposed to studying exosomes isolated from *in vitro* cell cultures. Yet, few studies so far have focused on body-fluid exosomes, and less so from those of human samples. Therefore, in this thesis I wanted to explore the immunological role of exosomes extracted from two immunologically active mucosal sites – the healthy and diseased lung and the mammary gland during lactation.

The presence of BALF-exosomes exhibiting a DC-exosome-like phenotype in lung of healthy individuals [226], suggests that they might have a role in local immune responses. Therefore, study I and II were conducted to explore this possibility by analyzing BALF-derived exosomes during health and during two separate inflammatory conditions, sarcoidosis (study I) and allergic asthma (study II).

The specific aims were to:

Study I: Characterize and functionally compare BALF isolated exosomes from patients with pulmonary sarcoidosis and healthy individuals.

Study II: Characterize and functionally compare BALF exosomes from birch sensitized asthmatic patients and healthy individuals.

Breastfeeding is known to protect against infections and might influence allergy development in the child. Differences in immune components in breast milk between mothers might lead to differences in immune modulation and allergic outcome of the child. Therefore, in study III we aimed to explore potential differences that might exist in the composition of breast milk-exosomes between mothers with regard to sensitization and lifestyle. Finally, due to the known protective role of breastfeeding against infections, in study IV we wanted to investigate the possibility of breast milk-exosomes being one protective factor against HIV-infection.

The specific aims were to:

Study III: Compare the composition of breast milk exosomes with regard to allergic status (IgE-sensitized or non-sensitized) or lifestyle (anthroposophic/non-anthroposophic) of the mother.

Study IV: Address whether human milk exosomes could prevent against HIV infection in DCs and subsequent trans-infection of T cells.

3 MATERIALS & METHODS

3.1 STUDY POPULATIONS

3.1.1 SARCOIDOSIS PATIENTS – STUDY I

All individuals included in this study were recruited from the Karolinska University Hospital, Stockholm, Sweden. Sarcoidosis patients were subjected to bronchoscopy and bronchoalveolar lavage (BAL) as part of a routine investigation for sarcoidosis. Diagnosis of the disease was established by clinical manifestations, radiological findings, analysis of BAL cells with elevated levels of $CD4^+/CD8^+$ T cell ratio and presence of granulomas by histology of bronchoscopic biopsy specimen. All healthy individuals were non-smokers, had normal chest X-rays and no previous respiratory infections for at least one month prior to BAL.

3.1.2 BIRCH ASTHMATICS – STUDY II

This work included mild birch asthmatics and healthy individuals all recruited from the Karolinska University Hospital. All patients had birch pollen-specific IgE in serum (≥2.0 kU/L) whereas controls were negative (≤0.35 kU/L). Patients were free of asthma exacerbations and lung infections at least 4 weeks prior to BAL with occasional medication with β2-agonist. BAL was performed according to established protocols [254] and was collected once from healthy controls and twice from asthmatics (before and after allergen challenge) with a minimum of 3 weeks between first- and second BAL.

3.1.3 ANTHROPOSOPHIC AND ALLERGEN SENSITIZED MOTHERS – STUDY III

This study was based on the prospective birth cohort study ALADDIN (Assessment of Lifestyle and Allergic Disease During INfancy) [255]. Breast milk was collected at 3-8 days and at 2 months after delivery. Mothers were characterized as anthroposophic or non-anthroposophic according to answers to a questionnaire. IgE-sensitization was determined if IgE-levels were ≥0.35 kU/l for the parents measured by Phadiatop® (kindly sponsored by Phadia AB) and for the children at 2 years of age by ImmunoCAP® in at least one of the seven allergens.

3.1.4 BREAST MILK AND BLOOD FROM HEALTHY INDIVIDUALS – STUDY IV

This study included human samples (breast milk, plasma and PBMCs) from healthy individuals. Human breast milk was collected at home from healthy volunteers that were non-smokers, had vaginal deliveries at full-term and had normal birth weight infants. Plasma and buffy coats for PBMC isolation were collected from healthy blood donors and were recruited from the Karolinska University Hospital.

3.1.5 ETHICAL PERMISSION

The local ethics committee approved all studies included in this thesis and all subjects participated voluntarily and provided written informed consent.

3.2 METHODS

The listed methods below were used in study I-IV of this thesis. A description of the following methods can be found in the respective publications.

- ✓ Bronchial epithelial cell culture (study I & II)
- ✓ Bronchoalveolar lavage (BAL) (study I & II)
- ✓ Confocal microscopy (study IV)
- ✓ Cytospin and May-Grünwald-Giemsa (study II)
- ✓ Electron microscopy (study I)
- ✓ Enzyme-linked immunosorbent spot (ELISPOT) (study I)
- ✓ Endotoxin test (study I)
- ✓ Enzyme-linked immunosorbent assay (ELISA) (study I & II)
- ✓ Exosome isolation with ultracentrifugation and immunoadsorption (study I-IV)
- ✓ Flow cytometry analysis (study I-IV)
- ✓ Generation of monocyte-derived dendritic cells (MDDCs) (study IV)
- ✓ High performance liquid chromatography (HPLC) (study II)
- ✓ Mass spectrometry analysis (study I)
- ✓ Silver staining of gel (study I)
- ✓ Statistical analysis (study I-IV)
- ✓ Sucrose gradient fractionation (study I & IV)
- ✓ Western blot analysis (study I & II)

4 RESULTS AND DISCUSSION

4.1 PRO-INFLAMMATORY EXOSOMES IN SARCOIDOSIS (STUDY I)

Various studies of sarcoidosis, including genetic studies and cell and cytokine analysis in BALF, have provided important insights into the mechanisms involved in the disease [201, 256]. However, we still do not fully understand the immunological events involved in the pathogenesis of sarcoidosis, partly due to the lack of a relevant animal model.

In 2003, Admyre *et al* reported the presence of exosomes carrying immune-relevant molecules in BALF from healthy individuals [226], suggesting a role of exosomes in local immune defenses. Consecutive studies in mice have reported diverse functions of BALF exosomes, with studies showing a pro-inflammatory [226] or tolerogenic effect [224]. These studies imply that exosomal function varies with regard to the biological context of the lung. At the time that our study was initiated, there were no available data on the function of BALF exosomes in humans. Yet, based on data from the murine system, we hypothesized that human BALF exosomes from healthy individuals might be functionally different compared to exosomes from BALF of patients suffering from lung inflammation. Thus, we sought to determine the function of BALF exosomes from healthy individuals and sarcoidosis patients in the context of inflammation.

Patients with pulmonary sarcoidosis exhibit an enhanced cellular infiltration and cell activation in BALF [257, 258], with potential effects on exosomal release as a consequence. Therefore, we hypothesized that levels of exosomes might differ in BALF from sarcoidosis patients compared to healthy controls. Accordingly, we found a significant increase in BALF exosomes in patients compared with healthy individuals (study I, figure 1A). In addition, we found an aberrant increase in several proteins on exosomes from patients compared to healthy controls, such as HLA-DR (study I, fig 2B and 4C), CD9, CD54 (study I, fig 2D and E) and HSP70 (study I, fig 4C). Taken together, we show that BALF exosomes from sarcoidosis patients are altered with regard to quantity as well as protein composition compared to those from healthy subjects.

By mass spectrometry analysis we identified the presence of neuregulin-1 (NRG1) on BALF exosomes (study I, Table 2) and by Western blot we confirmed an enrichment of this molecule in BALF exosomes from patients compared to exosomes from healthy controls (study I, fig 4C-D). In the human lung, NRG1 has been reported to function as a growth factor for pulmonary epithelial cells [259]. In addition, patients with acute lung injury display elevated levels of NRG1 in BALF and levels correlated with markers of inflammation [260]. Whether these results can be extrapolated to patients with other inflammatory diseases is not known. However, it is tempting to speculate that NRG1-expressing BALF exosomes could function as a new marker of inflammation and disease severity in sarcoidosis.

To study a potential immune function of BALF exosomes we exposed PBMCs from patients and healthy controls to BALF exosomes in an autologous setting, and measured the production of IL-13 and IFNγ as a readout of inflammation (study I, fig 5A-B). We found that PBMCs from patients produced significantly higher levels of IL-13 and IFNγ in response to BALF exosomes compared to those from healthy individuals, suggesting a pro-inflammatory function of exosomes from patients. Furthermore, due to the crucial involvement of airway epithelium in the initiation of immune responses in the lung [261], we next wanted to study the immune-modulatory effects of BALF exosomes on the bronchial epithelial cell line (BECs) 16HB14. We found that these cells released significantly higher levels of the pro-inflammatory cytokine IL-8 in response to exosomes from patients compared to those from healthy individuals (study I, fig 5C-D). Thus, our data suggests a pro-inflammatory function of BALF exosomes from patients with the ability to engage in both innate- and adaptive immune responses (see fig 4).

Further studies could help elucidate the mechanism of exosome-mediated immune activation in PBMCs observed in this study. We speculate that BALF exosomes carrying MHC-peptides could present antigens to T cells and thereby evoke an inflammatory response. Alternatively, they could evoke antigen-specific responses by transferring antigens and/or peptide-MHC molecules to recipient APCs in order to amplify an immune response [114, 115]. The mechanism by which BALF exosomes induce IL-8 in BECs is probably different from that in PBMCs. The striking similarities in clinical manifestations of tuberculosis and sarcoidosis, together with the finding of mycobacterial DNA in sarcoidosis samples [196], strongly suggest that MTB infection may function as an etiological agent of sarcoidosis (at least in a subgroup of patients). Previous studies have demonstrated the presence of mycobacterial components on BALF exosomes from *M. bovis*-infected mice with the ability to induce TNFα responses in naïve macrophages [229]. Thus, we hypothesize that BALF exosomes from sarcoidosis patients might carry pathogen-derived molecules that could induce activation in BECs through PRRs.

The up-regulation of HSP70 on BALF exosomes from patients compared to healthy controls is intriguing. Heat shock proteins are mainly located in the cytosol where they help maintain homeostasis by functioning as molecular chaperons. However, during abnormal conditions, such as during malignancies, HSP70 is upregulated and aberrantly located at the plasma membrane and can exert immunogenic functions [262]. Mycobacteria-infected macrophages have been shown to release exosomes enriched in HSP70, particularly at the exosomal membrane compared to exosomes from non-infected cells. Accordingly, exosomes enriched in HSP70 could trigger TNFα release at similar levels as those obtained by soluble HSP70, suggesting that HSP70 containing exosomes can function as potent inducers of inflammation [263]. In our study, we did not determine whether HSP70 was present at the surface or within BALF exosomes. However, it appears plausible that the aberrant expression in HSP70 in exosomes from patients might contribute to their pro-inflammatory functions.

Taken together, this is the first study reporting a function of BALF exosomes from humans. Furthermore, we provide firm evidence of a pro-inflammatory function of BALF exosomes from sarcoidosis patients. These studies could help pave the way for future studies exploring the role of exosomes in sarcoidosis pathology and/or the involvement of lung exosomes in other pulmonary inflammatory diseases.

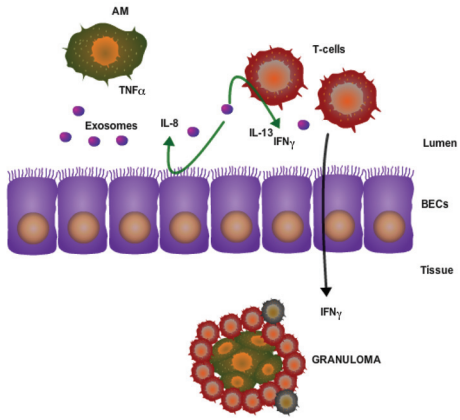

Figure 4. Suggested role of BALF exosomes as promoters of lung inflammation in sarcoidosis.
During sarcoidosis inflammation there is an increased release of pro-inflammatory exosomes that can stimulate IL-8 production in BECs, and IFNγ and IL-13 in T cells, which may contribute to granuloma formation. AM: alveolar macrophages, BECs: bronchial epithelial cells.

4.2 EXOSOMES AS MEDIATORS OF LEUKOTRIENE-PRODUCTION IN ASTHMA (STUDY II)

In study I, we found that exosomes in BALF from sarcoidosis patients exhibit pro-inflammatory functions *in vitro.* Therefore, we wanted to study how these results could be compared to other inflammatory diseases in the lung. We therefore aimed to study BALF exosomes in allergic asthma – a chronic inflammatory disease driven by a distinct set of inflammatory cells and cytokines compared to sarcoidosis inflammation.

In contrast to our findings on BALF exosomes isolated from sarcoidosis patients, we found no difference in the levels of exosomal protein in BALF of healthy individuals compared to those with allergic asthma, neither before nor after allergen challenge (study II, fig 1E). This is in accordance with our results showing no difference in cell numbers in the BALF between healthy subjects and asthmatics (study II, figure 1D), suggesting a limited allergic reaction. Nevertheless, we found enrichment in several exosomal-proteins in BALF from patients compared to those from healthy individuals, both at steady state and after allergen challenge (study II, fig 2A-F). This suggests an intrinsic alteration in exosome composition in BALF of patients with allergic asthma.

Previous studies by Esser *et al* found functional leukotriene (LT)-producing enzymes in exosomes from human DCs and macrophages [264]. LTs are key mediators of inflammation, and therefore, we wanted to investigate whether these enzymes were present in BALF exosomes. Accordingly, BALF exosomes from asthmatics and healthy subjects contained abundant levels of several LT-enzymes, including LTA_4H and LTC_4S (study II, Fig 4A). Most importantly, these enzymes were functional (study II, fig 4B) and had similar capacities for conversion of LTA_4 as APC-derived exosomes [264]. However, BALF exosomes had a higher LTA_4H activity relative to LTC_4S, while the opposite was found for APC-derived exosomes [264], suggesting that BALF exosomes might originate from other cell types than APCs.

To gain insights into the function of BALF exosomes in asthma, we made use of the BEC line 16HB14, which we exposed to BALF exosomes from asthmatics or healthy controls and subsequently measured the release of LTs and IL-8. We discovered that BECs produced significantly greater levels of IL-8 and LTs (mainly LTC_4) in response to BALF exosomes from asthmatics compared to controls, with no difference before and after allergen challenge (study II, fig 5A-B). This further reinforces an inherent or latent inflammatory activity of exosomes from patients. Furthermore, the induction of LTs in response to BALF exosomes from patients could be efficiently reduced upon treatment with the $CysLT_1$ receptor antagonist montelukast (study II, fig 5C), which suggests that exosome-induced IL-8 production I BECs is at least partly mediated by CysLTs. Taken together, these results imply that exosomes in BALF might assist in the production of pro-inflammatory cytokines and LTs in airway epithelial cells and thereby promote lung inflammation (see fig 5).

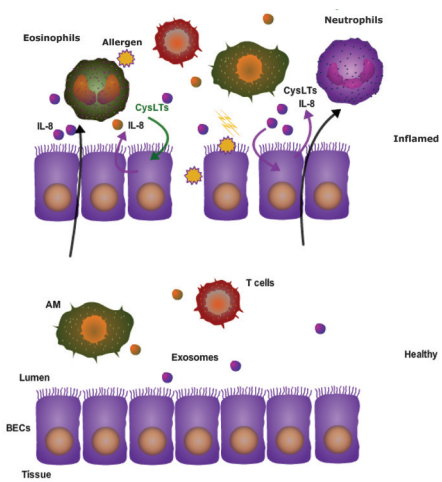

Figure 5. Suggested role of BALF exosomes in the pathogenesis of allergic asthma. During inflammation, exosomes are able to induce IL-8 and CysLT-production in BECs, which further helps in the recruitment of inflammatory cells to the lung. AM: alveolar macrophages, BECs: bronchial epithelial cells. Upper panel; inflammatory condition. Lower panel; healthy condition.

In contrast to our results, a recent study using a murine OVA-model of allergic airway inflammation found that exosome secretion, as well as total cell count was higher in BALF of asthmatic mice compared with controls [142]. Since our study included patients with mild asthma, we speculate that more severe cases of allergic asthma could potentially yield an increase in exosome production relative to non-inflammatory conditions. Nevertheless, our results show detectable differences in exosomal protein composition between asthmatics and healthy individuals, even in the absence of clinical symptoms. Previous studies have confirmed that even asthmatics with a mild form of airway inflammation show features of lung inflammation at steady state, such as increased levels and activation state of airway eosinophils and T cells, increased epithelial shedding, increased frequencies of hypodense macrophages and enlarged thickness of the basal membrane [265-267]. Thus, the altered exosome phenotype in patients during "steady state" could be due to an ongoing subclinical lung inflammation.

The detection of functional LT-producing enzymes in BALF exosomes from healthy individuals supports a role for exosomes in lung immunity. In fact, previous studies on tracheobronchial epithelium-derived exosomes have shown their ability to neutralize influenza virus via sialic acid moieties [141], suggesting the involvement of exosomes in regulating host defense reactions in the lung. Our study implies that LT generation by BALF exosomes from healthy individuals might constitute an additional mechanism for efficient immune responses against infectious agents. In the context of host defense, large amounts of LTA_4 produced by infiltrating neutrophils could potentially be metabolized into LTs by LT-producing enzymes present in BALF exosomes and thus facilitate pathogen clearance. Although human exosomes from plasma also carry functional LT-producing enzymes [264], we detected 50 times more LTs from LTA_4 generated by BALF exosomes compared to those isolated from plasma. This further emphasizes the relevance of BALF exosomes in lung immunity in healthy individuals.

Not all cells are equipped with all the cascade enzymes necessary for LT production. Despite this, cells expressing either LTA_4H or LTC_4S have the potential capacity to produce LTs due to the intercellular transfer of LTA_4. This phenomenon called "transcellular metabolism" has been reported for several cell types including human airway epithelial cells [268]. In addition, further studies have shown that human BECs express an active- and inducible 5-LO pathway upon activation and can generate *de novo* LT-synthesis [269]. This corroborates our findings on LT-production in BECs and we further show that BALF exosomes from asthmatics boosts LT production in these cells. How exosomes induce LT-synthesis in BECs is currently not known. However, we hypothesize that transfer of LT-enzymes to BECs via exosomes could potentiate their LT-synthesizing capacity. Alternatively, BALF exosomes could potentially induce the expression of LT enzymes in BECs by delivering pro-inflammatory stimuli to the cells.

One of the caveats with this study is the limited number of patients and the fact that exosomes from BALF were isolated at one single time point after allergen challenge. Asthmatics were subjected to BAL 24 h after allergen provocation, which likely reflects a resolution phase of the inflammation [270]. In fact, previous analysis of BALF

cells and cytokines from the same individuals exhibited an increase in $CD4^{+}Foxp3^{+}$ T cells and IL-10 at this particular time-point [271]. This suggests that anti-inflammatory mechanism have already been initiated at 24 h in order to counteract inflammation. Therefore, the lack of phenotypic and functional differences seen between BALF exosomes from asthmatics before and after allergen challenge could be due to the late time-point of choice. Explorations of other time-points may therefore reveal novel functional differences between BALF exosomes from asthmatics before and after allergen challenge. Nonetheless, this is the first study characterizing BALF exosomes in allergic asthma and adds to our knowledge about the role of exosomes in the human lung.

4.3 BREAST MILK EXOSOMES IN RELATION TO LIFESTYLE AND ALLERGIC SENSITIZATION (STUDY III)

Both environmental and genetic factors are known to strongly influence allergy development [272, 273]. Environmental exposures pre-natally and/or during early childhood, including diet, are thought to play a vital role on allergic outcome later in life [273]. Breastfeeding has long been known to provide immune-related benefits to the child [242]. As a result, extensive research has attempted to establish a relation between breastfeeding and allergy development in the child. However, the results obtained have been controversial, with studies reporting a protective effect of breast-feeding on allergic outcome, while others support a promoting, or a neutral effect [249]. Notably, differences in the immune profile of breast milk have been reported between mothers with regard to maternal allergies [274, 275] and environmental exposures [276]. Therefore, it has been postulated that at least part of the discrepancies between the outcomes could be attributed to individual variations in immune content of breast milk.

Previous studies have shown that exosomes isolated from human breast milk have the ability to modulate immune responses *in vitro* [145], suggesting a potential impact on the child's immune system. Here we aimed to address potential phenotypic differences in exosome composition in breast milk from mothers with regard to allergic sensitization. In addition, we wanted to address whether differences in maternal lifestyle, which implies differences in environmental exposures, could also affect exosome composition. For this purpose we chose to study the anthroposophic lifestyle since this way of living has previously been associated with a reduced risk of developing allergies in children [248, 255, 277, 278]. Ultimately, we sought to determine whether differences in exosomal phenotype in the mother's breast milk associate with allergic sensitization of the child at 2 years of age.

Breast milk comprises a mixture of different cell types [237]. Accordingly, our data suggest that human breast milk also contains phenotypically different subpopulations of exosomes. We approached the analysis by immunoprecipitating exosomes using beads coated with anti-MHC class II or anti-CD63 antibodies. This isolation method allowed us to precipitate exosomes with different ratios of CD63/MHC class II expression as seen by flow cytometry, thus supporting the existence of phenotypically different exosomes in breast milk (study III, fig 2C) (see fig 6).

We then analyzed the expression of several surface proteins on subpopulations of exosomes from mothers with regard to sensitization and/or lifestyle (study III, fig 3). We found that allergen-sensitized mothers had a decrease in MUC1 expression on exosomes bound to anti-CD63-coated beads compared to those from non-sensitized mothers (study III, fig 3H). Strikingly, the same molecule was decreased on exosomes from anthroposophic versus non-anthroposophic mothers, but this was only apparent when analyzing exosomes selected on anti-MHC class II-coated beads (study III, fig 3L). In addition, the tetraspanin CD63, which is highly enriched on breast milk-exosomes, was found to be upregulated on CD63-selected exosomes from anthroposophic mothers compared to non-anthroposophic women (study III, fig 3M). Since flow cytometry analysis of exosome-coated beads does not allow for exosome quantification, differences in the expression of surface molecules on exosomes may reflect an increase/decrease in the numbers of exosomes expressing that particular molecule, or a selective enrichment/decrease of surface molecules on exosomes. Nonetheless, our data suggests that maternal sensitization and lifestyle influences the phenotype of different subpopulations of exosomes in human breast milk.

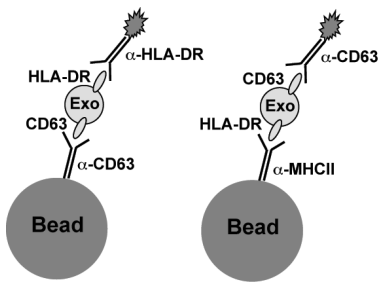

Figure 6. Isolation of exosomes on anti-CD63 latex beads or anti-MHC class II Dyna beads and detection by flow cytometry.

Next we wanted to correlate our results with sensitization outcome of the breast-fed children. To our surprise, we found that children that developed allergic sensitization at age 2 had been breastfed by mothers that exhibited significantly higher levels of MHC class I on exosomes bound to anti-CD63-coated beads (study IV, fig 4C). While these observations are interesting, further studies are required to elucidate a potential effect of these phenotypic observations on allergic outcome. Furthermore, despite detected differences in MUC1 expression on milk-exosomes with regard to maternal sensitization and lifestyle, we were unable to detect an association between MUC1 expression and allergic-sensitization in the child (study III, fig 4D). However, the low number of children who developed IgE-sensitization in our study could partly explain the lack of association. Thus, future studies including a bigger group of children could help address this.

The finding that MUC1 levels were altered on exosomes in mothers with respect to sensitization and lifestyle is intriguing. However, the potential immune-effects of these phenotypic alterations are currently elusive. Secreted MUC1 in human milk is known to be involved in pathogen binding, and thereby serving protective functions against infections [279, 280]. Thus, we speculate that MUC1-bearing exosomes in breast milk could function as decoy receptors for pathogens and facilitate their clearance. Accordingly, the lower expression of MUC1 on exosomes from anthroposophic mothers fits well with the hygiene hypothesis, which postulates that early exposure to infectious agents protects against allergy development later in life [281]. Thus, if allowed to speculate, children breastfed by anthroposophic mothers would receive less MUC1 on exosomes through breast milk, which could render them more susceptible to infections and less likely to develop allergies as a consequence. In addition to the reported role of MUC1 on pathogen binding, it has also been described as a ligand for the adhesion molecule CD54 [282]. Therefore, MUC1-expressing exosomes could potentially mediate cell interactions with CD54-expressing immune cells in the gut or endothelial cells, which could constitute a mechanism to enter systemic circulation.

The finding that CD63 was significantly increased on exosomes from anthroposophic- compared to non-anthroposophic mothers is interesting due to the important roles of tetraspanins in the immune system [283]. For instance, tetraspanins provide organization of proteins and are thought to facilitate interactions with important immune-related molecules, such as MHC class II [284]. However, little is known about the role of tetraspanins on exosomes but studies have suggested a function in antigen-presentation, exosome production [284] and mediating cell-targeting [285].

Taken together, our results provide valuable insights into how maternal sensitization and lifestyle might affect the exosome profile in human breast milk. Nevertheless, we are far from fully understanding how these results relate to outcome of allergic sensitization in the child. Hence, future studies exploring the biological function of MUC1-bearing exosomes and the function of phenotypically different subpopulatios of exosomes will be vital to reveal these complicated relations.

4.4 HUMAN BREAST MILK EXOSOMES INHIBIT HIV INFECTION OF DCs (STUDY IV)

Despite the health benefits associated with breastfeeding, breastfeeding by HIV-positive mothers is highly controversial since HIV can be transmitted to the child through breast milk [286]. Surprisingly, a number of studies have reported a significantly lower risk of HIV infection in exclusively breastfed- versus mixed- or formula-fed children [287, 288], pointing towards a protective effect of breast milk against HIV-infection. In fact, several components in milk have been suggested to mediate protection against HIV. One example is MUC1, which has been shown to compete with HIV for binding to DC-SIGN on DCs, resulting in the inhibition of HIV transfer to T cells [289]. DCs can, however, also be productively infected by HIV by exploiting innate signaling through DC-SIGN [27], among other mechanisms [290].

The presence of MUC1 on exosomes from human breast milk (study III) led us to speculate that milk-exosomes could have an effect on HIV infection in DCs and or *trans*-infection of T cells. As a comparison we used exosomes from another body fluid, namely plasma-derived exosomes. We conditioned MDDCs with milk-exosomes or plasma-exosomes for 1 h prior to infection with HIV-1_{BaL} for 4 h followed by extensive wash and incubation at 37°C. For viral transfer experiments, MDDCs pre-exposed to exosomes and HIV-1_{BaL} were thereafter co-incubated with allogeneic $CD4^+$ T cells. Subsequently, MDDCs or T cells were analyzed for productive infection (p24+) by flow cytometry after 5 days in culture.

We found that breast milk exosomes, but not plasma exosomes, significantly reduced productive infection of MDDCs (study IV, fig 1B-D). Moreover, MDDCs that were pre-conditioned with milk-exosomes, but not with plasma-exosomes, were significantly less efficient in *trans*-infecting T cells (study IV, fig 2). Therefore, these results suggest that milk-exosomes act on DCs, thereby suppressing DC infection and subsequent infectivity of T cells.

To gain insights into exosome-DC interaction, we labeled exosomes with a lipid dye and measured uptake by MDDCs by flow cytometry and confocal microscopy at different time points (30 min, 1 h, 2 h and 4 h). By confocal microscope analysis, we could determine that exosomes accumulated readily and most prominently inside the cell already after 2 hours (study IV, fig 3A). However, by flow cytometry analysis (a quantitative and more sensitive detection method), we found detectable levels of exosome-uptake/binding already after 30 min, with the most notable interaction occurring at 1 h (study IV, fig 3B). In contrast, no uptake of plasma-exosomes could be detected up to 4 h (study IV, data not shown). In conclusion, these results show that milk-exosomes are efficiently taken up by MDDCs and suggests a selective uptake of milk-exosomes, since no uptake was observed with plasma-exosomes within this time frame.

DC-SIGN and adhesion molecules, such as CD54, have previously been shown to mediate interactions with HIV [291, 292]. Based on this, we reasoned that both DC-SIGN and CD54 might constitute potential binding and entry-points for exosomes to cells, and could provide an explanation to the blocking effect of milk-exosomes on HIV infection. Therefore, to study the contribution of these receptors in exosome-uptake, we blocked DC-SIGN and/or CD54 on DCs and subsequently measured exosome-uptake by confocal microscopy and flow cytometry analysis. We found that cells treated with isotype control or CD54 antibodies efficiently took up exosomes, whereas blocking DC-SIGN on DCs completely abolished exosome-uptake, seen by confocal microscopy analysis (study IV, fig 4A). Nevertheless, by flow cytometry, we detected a certain degree of exosome uptake in DCs blocked with DC-SIGN at 30 min post incubation, albeit at lower levels compared to isotype- and CD54 treated cells (study IV, fig 4B). Of note, the difference between anti-DC-SIGN and anti-CD54-treated cells was reduced over time, possibly due to uptake via alternative routes and/or internalization and recycling of DC-SIGN to the cell-surface [293].

The notion that exosomes can interact with cells has been well documented. However, the mode of interaction still remains unclear. Studies suggest that phagocytic cells mainly take up exosomes through phagocytosis, whereas non-phagocytic cells interact with exosomes at the cell-surface [294]. Additional studies by Morelli *et al* further reinforce the notion that exosomes are internalized efficiently by phagocytic cells, including DCs [111]. In line with these observations, we found that MDDCs were capable of internalizing milk-exosomes, while incapable of internalizing plasma-exosomes within the time-span of 4 h. Therefore, our data suggest that the mode of interaction between phagocytic cells and exosomes might depend on the type of exosomes, and could potentially involve surface binding [124], direct fusion [295, 296], and/or internalization. We report a previously unknown mode of interaction between exosomes and DCs involving the binding of DC-SIGN. However, whether this particular interaction mediates the internalization of milk-exosomes is not clear. Nevertheless, as seen by confocal microscopy analysis, blocking of DC-SIGN led to reduced exosome internalization in MDDCs (study IV, fig 4A), suggesting a role of DC-SIGN in exosome-uptake. However, other routs of entry are likely also involved since blocking of DC-SIGN resulted in partial inhibition in exosome-binding/uptake.

We speculate that MUC1 on exosomes might be mediating interactions with DC-SIGN. In fact, DC-SIGN has previously been reported to recognize and bind Lewis X structures present on MUC1 [289]. However, we cannot exclude the contribution of other DC-SIGN ligands that might be present on exosomes, such as other glycans containing similar binding motifs. We further postulate that the protective effect of milk-exosomes on HIV-infection is, at least partly, DC-SIGN mediated. We speculate that this could occur by the ability of milk-exosomes to: 1) outcompete HIV for the binding of DC-SIGN, 2) target DC-SIGN for lysosomal degradation, and/or 3) induce DC-SIGN signaling with potential effects on DC-function. However, it is possible that additional mechanisms, such as the binding of exosomes to other co-receptors for HIV entry, such as CCR5, could account for the protective effect. Also, other non-specific mechanisms, such as induced maturation of DCs by milk-exosomes, could potentially account for a lower infectivity rate. In fact, previous studies suggest that mature DCs are less vulnerable to HIV infection [297]. Yet, we found that DCs expressed similar levels of the maturation marker CD86 before- and after exosome-exposure, which would argue against such a mechanism. In contrast, T cell activation seems to be a major factor facilitating HIV-infection in these cells [298]. Thus, we cannot exclude an indirect effect of exosomes by affecting T cell activation. However, since we washed DCs prior to co-culture with T cells, this explanation appears unlikely since the washing step likely removes any unbound exosomes. However, future studies are required to address the underlying mechanisms.

Studies have shown that HIV infected cells can secrete exosomes that carry viral proteins and viral-derived small RNAs [299], which might be important for mediating viral spread. Therefore future studies will have to address whether HIV-positive mothers carry viral-containing exosomes in their breast milk. Furthermore, revealing the ratio of HIV-containing exosomes versus HIV-negative exosomes in breast milk of

HIV positive mothers will be helpful in trying to decipher the overall effect of milk-exosomes on HIV transmission from mother-to-child.

In summary, our data show that breast milk-exosomes inhibit productive HIV infection of MDDCs and subsequent *trans*-infection of $CD4^+$ T cells. Furthermore, we show that exosomes bind to DCs through a mechanism that is partly dependent on exosome-DC-SIGN interactions. Therefore, we suggest that milk-derived exosomes may play a role in lowering the risk of HIV-1 transmission by breastfeeding. We propose a mechanistic model in which exosomes compete with HIV for the binding to DC-SIGN and thereby provide protection against HIV infection in DCs and *trans*-infection to T cells (see fig 7).

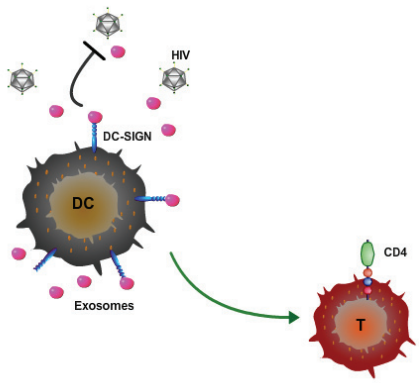

Figure 7. Proposed mechanism for breast milk exosome-mediated protection against HIV-infection. Exosomes with MUC1 on the surface bind to DC-SIGN on DCs and thereby block entry of HIV and subsequent viral transfer to T cells.

5 CONCLUSIONS

Study I: This study shows that patients with sarcoidosis have increased production of exosomes in their lungs compared to healthy individuals. These exosomes have the ability to stimulate cytokine production from autologous PBMCs and epithelial cells, suggesting both an adaptive and an innate mode of activation. These results suggest that exosomes from the BALF of patients with sarcoidosis might have a role in the pathogenesis of the disease and could be of relevance for the development of novel treatment strategies for sarcoidosis, but also for the development of new disease and prognostic markers for sarcoidosis.

Study II: We show that BALF-derived exosomes from patients with allergic asthma have an altered exosome profile compared to healthy individuals, and that they exert pro-inflammatory functions by inducing the release of IL-8 and CysLTs in bronchial epithelial cells. Thus, our data suggest that BALF exosomes might contribute to asthma pathogenesis by promoting pro-inflammatory cytokines and LT generation in the lung. This could have implications in the development of future therapies targeting exosome production.

Study III: In this study we found that human breast milk exosomes differ phenotypically between mothers with respect to their allergic sensitization and lifestyle. Therefore we suggest that changes in exosome-composition might differentially influence the immune system of the newborn with possible effects on allergy development as a consequence.

Study IV: We show that pre-exposure to human breast milk derived exosomes reduces productive HIV-1 infection of monocyte-derived DCs and subsequent viral transfer to $CD4^+$ T cells. This suggests that milk-exosomes might be one of the components that confer protection from HIV-1 transmission from mother to child through breastfeeding.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kroppen består av olika celler som i ett strukturerat nätverk utgör skilda organen i kroppen, såsom lungor och tarm. För att organen ska kunna fungera normalt, så är det ytterst viktigt att cellerna kommunicerar med varandra. Denna kommunikation kan ske via beröring mellan celler som kräver direktkontakt. Det kan dock finnas situationer då det är omöjligt, eller rent av opraktiskt att behöva befinna sig på samma plats för att utbyta information. Ett utmärkt exempel på detta är kommunikationen mellan oss människor. Att kunna ses och skaka hand kan vara ett effektivt sätt att kommunicera, men omöjligt att utföra mellan två personer belägna på olika platser i världen. Däremot har vi möjligheten att kunna kommunicera via telefon, email, brev, Skype etc. Även vissa celler har utvecklat en rad mekanismer för att kunna kommunicera sinsemellan, trots att de är belägna vid olika platser i kroppen. Ett exempel på detta är exosomer. Alla celler i kroppen kan frisätta exosomer, mycket små sfäriska budbärare med förmågan att transportera information från en cell till en annan. Fördelen med exosomer gentemot att frisätta enstaka proteiner, såsom hormoner, är att exosomer kan bära på 100-tals molekyler som tillsammans kan leverera ett mer sofistikerat kommunikationsutbyte. Jag föreställer mig hormoner som ord ur en konversation, medan exosomer utgör hela meningar.

Betydelsen av exosomer vid sjukdom och hälsa har gradvis börjat belysas. Man har hittills påvisat allt från sjukdomsalstrande till tolerans-inducerande funktioner, vilket tyder på en relativt komplex kommunikationsdynamik. Forskare har upptäckt att exosomer från olika celler kan innehålla olika typer av information, vilket delvis förklarar exosomernas skilda effekter. Exosomer har kunnat isoleras från alla mänskliga kroppsvätskor man analyserat hittills, som t ex. saliv, bröstmjölk, blod och urin, vilket tyder på en viktig funktion i kroppen. Genom att studera exosomer från kroppsvätskor hoppas vi kunna få en ökad förståelse för exosomers fysiologiska funktion.

Vid varje andetag så exponeras lungorna för en mängd både ofarliga- och potentiellt skadliga faktorer. Av den anledningen är lungan utrustad med ett kraftfullt immunförsvar som kan skydda kroppen mot infektioner, men som även kan urskilja farligt från ofarligt. Trots detta händer det att immunsystemet attackerar harmlösa ämnen, eller förlorar striden mot sjukdomsalstrande virus eller bakterier, vilket leder till kronisk inflammation. En hypotes är att exosomer utgör små budbärare i lungan som kan leverera ”goda” nyheter under normala situationer, eller ”dåliga” nyheter under en inflammation. I arbete I och II studerade vi exosomer isolerade från lungvätska från patienter med sarkoidos och astma, två inflammationssjukdomar i lungan. Vi fann att exosomerna hade sjukdomsalstrande egenskaper och kunde jämfört med exosomer från friska individer bl.a. inducera en ökad grad av inflammatoriska substanser hos lungceller.

Studier har tidigare klarlagt att amning kan skydda spädbarnet mot infektioner, och ha en eventuell skyddseffekt mot allergier. Resultaten angående allergiutveckling har däremot varit tvetydiga. En tidigare studie har visat att mammans bröstmjölk kan

variera i immunologiskt innehåll, vilket delvis kan förklara de motstridiga resultaten mellan olika studier. Vi har tidigare visat att exosomer i bröstmjölk har förmågan att inducera så kallade T celler med regulatoriska funktioner vilket kan ha betydelse för induktion av tolerans hos det ammade barnet. I studie III påvisade vi skillnader i sammansättningen av exosomer i mjölk mellan kvinnor med hänsyn till moderns allergiska status och livsstil vilket vi tror kan ha effekter på allergiutveckling hos det ammade barnet.
I studie IV presenterar vi en ny kommunikationsmekanism mellan bröstmjölks-exosomer och dendritiska celler, vilket är en typ av cell som spelar en ytterst viktig roll i immunförsvaret. Vi fann att mjölk-exosomer bär på signaler som cellerna specifikt känner igen. Förvånande nog så överförde exosomerna ett ökat skydd hos cellerna mot HIV infektion jämfört med celler som inte hade varit i kontakt med mjölk-exosomer. Eftersom HIV-positiva mammor som ammar kan överföra HIV via mjölken, så skulle exosomer i bröstmjölk kunna utgöra en skyddande faktor mot HIV-infektion via amning genom att konkurrera med HIV för inbindning till immunceller.

Sammanfattningsvis så har studierna inkluderade i den här avhandlingen gett en ökad förståelse för hur exosomer isolerade från bröstmjölk och lungor kan medverka i immunologiska processer. Vi är dock fortfarande i ett tidigt stadium och fler studier behövs för att få full förståelse för hur exosomer fungerar och interagerar med celler i kroppen. Men framtiden ser ljus ut och exosomer skulle kunna erbjuda en ny sorts terapi mot olika typer av sjukdomar. Man skulle t.ex. kunna slå ut sjukdomsalstrande exosomer i lungan under en inflammation, eller främja produktionen av toleransinducerande exosomer i syfte att motverka sjukdomar, såsom allergier.

7 REFLECTIONS & FUTURE PERSPECTIVES

Our work has shown that the amount of BALF exosomes- their protein and function differ between patients with sarcoidosis and healthy controls (study I). In addition, our findings that BALF exosomes from sarcoidosis patients and individuals with allergic asthma (study II) are able to promote pro-inflammatory cytokine release *in vitro* in BECs, suggest a contribution of these vesicles in driving inflammation *in vivo*. However, the mechanism of action by BALF exosomes might be disease-context dependent due to differences in the cellular make-up of the lung in patients with different pulmonary inflammatory diseases. Hence, further studies are required to characterize functional similarities and disparities of BALF exosomes between different lung inflammatory diseases. In the future, pro-inflammatory exosomes could potentially be targeted for therapeutic purposes and further insights on BALF exosome function in the context of a given disease could help develop tailor-made treatments. However, BALF exosomes, likely contain a heterogeneous population of exosomes as seen for exosomes in breast milk (study III). These different exosomes might carry out different functions depending on their cellular origin, and could include exosomes with tolerogenic functions that help counteract disease. Therefore, further studies need to focus on characterizing subpopulations of exosomes and other extracellular vesicles in BALF in order to understand their overall contribution in inflammation. In addition, detailed characterization of BALF exosomes from sarcoidosis patients by proteomics or microarray analysis could provide new insights on the composition of exosomes and reveal new biomarkers of sarcoidosis.

It is well established that breast-feeding protects against enteropathic infections in the child [300] and has further been suggested to protect against other immune-related diseases, such as asthma, inflammatory bowel disease and multiple sclerosis [301-304]. Hence, it has been hypothesized that breast milk might confer protection against diseases by promoting antigen-specific tolerance in the breastfed child [305]. I therefore would like to explore a potential contribution of milk-exosomes in tolerance induction, both *in vitro* and *in vivo*. Furthermore, since breast milk likely contains different types of vesicles with potentially distinct or overlapping functions, I would like to investigate the immune-regulatory functions of two different vesicle-types in milk, exosomes and MVs.

7.1 PRELIMINARY RESULTS

Antigen-specific immune tolerance *in vivo* is thought to require antigen-presentation by “tolerogenic” DC in the mesenteric lymph nodes and following induction of antigen-specific Tregs [306]. I therefore would like to address whether exosomes and/or MV can influence DC-biology towards a tolerogenic function by monitoring their capacity to induce Tregs. In order to test the role of breast milk exosomes and MVs in a mouse *in vivo* model, we first had to isolate and characterize murine milk vesicles since it has not previously been done. Exosomes and MVs were isolated from milk collected from pup stomachs 12 days postpartum by differential ultracentrifugation

according to established protocols [79] with modifications, followed by EM analysis. Results show the presence of vesicles from the stomachs of nursed pups (Figure 8). We found an enrichment of smaller vesicles in the exoscme preparations with a size distribution of 50-100 nm in diameter and with the typical cup-shape morphology previously reported for exosomes (fig 8A). The MV preparations on the other hand contained larger vesicles that were generally bigger than 100 nm (fig 8B).

This is the first time that an attempt has been made to detect and discriminate between different vesicles in murine breast milk. Furthermore, the presence of vesicles in the milk collected from stomachs of pups, suggests that they remain relatively intact in this comparatively harsh environment. The cellu ar origin of the vesicles and whether they also can originate from the stomach epithelium of the pup has to be further examined.

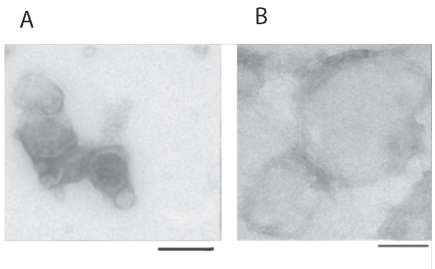

Figure 8. Vesicles from milk extracted from mouse pup stomachs. Exosomes (A) and MV (B) were purified from the stomachs of nursed C57Bl/6 wild type (WT) pups 12 days postpartum and analyzed by electron microscopy. Milk in the stomachs of pups from the same litter was pooled (3-8 stomachs per sample). One representative sample out of two is shown. Scale bar 100 nm.

To investigate the potential immunoregulatory effect of exosomes and MVs from mouse milk, we conditioned splenic $CD11c^+$ DCs with milk-exosomes or MVs for 24 h prior to co-culture with naïve $CD4^+$ T cells from OT-II mice. As a read-out of tolerogenicity we analyzed the capacity of conditioned-DC to increase the frequency of $Foxp3^+CD4^+$ T cells at day 4 of co-culture. Our *in vitro* preliminary data show that splenic $CD11c^+$ DCs exposed to milk-derived MVs for 24 h could dramatically increase the frequencies of $Foxp3^+CD4^+$ Tregs compared to DCs cultured alone or co-cultured with exosomes (fig 9A). This suggests that MVs carry regulatory signals that educate DCs to become "tolerogenic". Furthermore, we observed suppression in the proliferation of $CD4^+Foxp3^-$ T cells in the T cell co-cultures with MV-conditioned DCs compared to DCs cultured alone or DCs exposed to exosomes (fig 9B). We speculate that this suppression is mediated via $Foxp3^+$ cells.

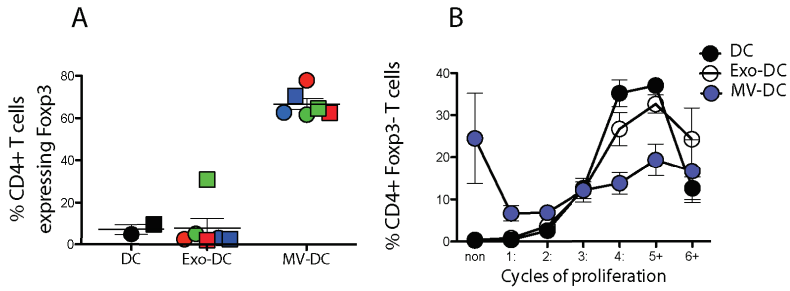

Figure 9. Tolerogenic effect of MV in milk. WT C57Bl/6 mice (n=3) were injected with FLT3L producing tumor cells (2-5 x 10^6 cells s.c. dorsum per mouse) in order to expand the DC population *in vivo*. 10-14 days later, $CD11c^+$ DCs from spleen were isolated by MACS sorting and cultured (2 x 10^5 cells per well) in 96 well plates alone (DC), with exosomes (Exo-DC) or microvesicles (MV-DC) (10 μg exosomes/MV per well) in exosome-depleted culture media supplemented with an ovalbumin (OVA) peptide (H2N-ISQAVHAAHAEINEAGR-OH, 500 nM), IL-2 (10 ng/mL) and TGFβ (2 ng/mL). After 24 h, the DCs were transferred to new 96 well plates and co-cultured with CFSE-labelled naive OVA-specific T cells (OT-II CD4+CD62L+) isolated by MACS sorting from the spleen of OT-II mice (DC-T cells ratio 1:2). After 4 days the cells were harvested for subsequent analysis by flow cytometry for A) Foxp3 expression (CD4+Foxp3+ T cells), and B) proliferation of CD4+Foxp3- cells in each division cycle. Three different exosomes and MV samples, each pooled from 3-8 pup stomachs from the same litter, were tested at two different experiments. A) Results are expressed as mean ± SEM. Each color (red, green and blue) represents one biological sample (exosomes or MV, n=3), and black, DC pre-cultured alone (n=2). Shapes correspond to different experiments (circles = experiment 1, squares = experiment 2). B) Results are expressed as mean ± SEM of DC (n=2), Exo-DC (n=6) and MV-DC (n=6) for each proliferative cycle.

Future experiments will focus on reproducing the presented preliminary results with the aim to reveal the biological changes in MV-conditioned DCs resulting in Treg induction. For this purpose, we aim to test the involvement of candidate genes by microarray in DCs exposed to MVs. Examples of relevant genes to examine are retinalcehyde dehydrogenase (RALDH2 enzyme), TGFβ and IL-10. Our results suggest that the $Foxp3^+$ population might be mediating inhibition of T cell proliferation. In order to directly test the suppressive activity of Tregs I will perform Treg suppression assays in *vitro* [307].

Gut DCs imprint CCR9 and α4β7 on T cells, which enables them to home to the small intestine in response to MAdCAM-1 and CCL25 in the gut [308]. It has further been shown that expression of both CCR9 and α4β7 is required for Tregs to localize to the gut and to induce oral tolerance [309]. Based on these results, it would be desirable to explore if MV-DCs can induce gut-homing receptors on T cells, in particular Tregs. If so, this could open up a new therapeutic avenue using MV-conditioned DCs as a potential vaccine against gut inflammatory diseases by driving Treg development and gut homing.

Allergic disease can be seen as a failure of the immune system to maintain tolerance to innocuous antigens. Oral tolerance to food antigens is established early in life, often during the breast-feeding period. Therefore, potential differences in immune-regulatory components of breast milk could have effects on the immune system of the child with subsequent effect on allergy development. Our results show phenotypic differences in breast milk exosomes between mothers with regard to sensitization to allergens and life-style (study III). These findings provide a guide towards future

research focusing on the differential "tolerogenic" function of milk-exosomes and MVs from mothers with regard to allergies or other immune-related diseases. Furthermore, the presence of phenotypically different sub-populations of exosomes in milk found in our study (study III) could be reflecting the existence of functionally different vesicles in breast milk, a research venue worth pursuing.

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